AN INVESTIGATION OF THE STOICHIOMETRY OF Na^+ COTRANSPORT WITH DOPAMINE IN RAT AND HUMAN DOPAMINE TRANSPORTERS EXPRESSED IN HUMAN EMBRYONIC KIDNEY CELLS

By

PAUL DIEDRICH SCHUMACHER III

A thesis submitted in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE IN CHEMISTRY

WASHINGTON STATE UNIVERSITY
Department of Chemistry

May 2001

Form SF298 Citation Data

Report Date *("DD MON YYYY")* 00052001	**Report Type** N/A	**Dates Covered (from... to)** *("DD MON YYYY")*

Title and Subtitle An Investigation of the Stoichiometry of Na+ Cotransport with Dopamine in Rat and Human Dopamine Transporters Expressed in Human Embryonic Kidney Cells	**Contract or Grant Number**
	Program Element Number
Authors Schumacher III, Paul Diedrich	**Project Number**
	Task Number
	Work Unit Number
Performing Organization Name(s) and Address(es) Washington State University Department of Chemistry	**Performing Organization Number(s)**
Sponsoring/Monitoring Agency Name(s) and Address(es)	**Monitoring Agency Acronym**
	Monitoring Agency Report Number(s)
Distribution/Availability Statement Approved for public release, distribution unlimited	
Supplementary Notes	
Abstract	
Subject Terms	
Document Classification unclassified	**Classification of SF298** unclassified
Classification of Abstract unclassified	**Limitation of Abstract** unlimited
Number of Pages 85	

To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of PAUL DIEDRICH SCHUMACHER III find it satisfactory and recommend that it be accepted.

_____
Chair

_____

_____

_____

ACKNOWLEDGEMENT

There are many individuals who have contributed to the development of this paper. I hope that some day I can repay all of them for the assistance they have provided.

My appreciation to Fred Schuetze and Duncan Vanderwall who provided unending technical and mechanical assistance during the development of the instrumentation used throughout the experiments.

To my fellow CLUB DAT members; Jo Anne Alfaro, Carrie Kaminski, Shannon George, Cortney Wright, Sherri Stark, and Myung Kim, I thank for all their time, energy and patience in dealing with me, helping with all experiments and the grammatical assistance in the writing of this paper.

To my mentors Dr. Jeremy Lessmann and Dr. James Schenk, I can not thank them enough for all the help, assistance, humor and advice throughout my education here at WSU. I would not have made it without them

Finally, I wish to thank my wife Lisa and my two sons Jeremiah and Joshua for being there when I needed them, listening to me talk about a subject they do not like and providing the emotional support to help me with this endeavor. I am forever grateful.

AN INVESTIGATION OF THE STOICHIOMETRY OF Na^+ COTRANSPORT WITH DOPAMINE IN RAT AND HUMAN DOPAMINE TRANSPORTERS EXPRESSED IN HUMAN EMBRYONIC KIDNEY CELLS

Abstract

by Paul Diedrich Schumacher III, M.S.
Washington State University
May 2001

Chair: James O. Schenk

The neuronal membrane transporter for dopamine (DAT) is a member of the Na^+ and Cl^- dependent family of transporters and concentrates dopamine intracellularly up to 10^6 fold over extracellular levels. Na^+ is hypothesized to provide the major driving force for this concentrative property. In order to validate the Na^+-gradient hypothesis, it must be shown that Na^+ accumulates in stoichiometric amounts along with the substrate.

Results herein show in human embryonic kidney (HEK) 293 cells expressing the rat DAT and HEK cells expressing the human DAT that: 1) dopamine stimulates transport of Na^+ as measured by flame photometry and the fluorescent sodium indicator, Sodium Green™; 2) the time course of accumulation of intracellular Na^+ is time-locked with dopamine; 3) the amount of Na^+ accumulated intracellularly is dependent on the extracellular concentration of dopamine; 4) dopamine-stimulated accumulation of Na^+ is blocked by both cocaine and mazindol; and 5) the mass of Na^+ accumulated when related to dopamine was found to be 2.6 ± 0.2 SD and 2.5 ± 0.2 SD for rat DAT and human DAT, respectively, when estimated by fluorescence spectroscopy and

1.9 ± 0.1 and 1.9 ± 0.2 for rat DAT and human DAT, respectively, when estimated by flame photometry indicating a stoichiometric coefficient for Na^+ transport with dopamine is around 2.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER TWO

Page

LIST OF FIGURES

CHAPTER ONE

Page

CHAPTER TWO

LIST OF EQUATIONS

CHAPTER ONE

Page

CHAPTER TWO

ATTRIBUTIONS

The text of this thesis is in the format required for submission to the *Journal of Neurochemistry* for publication. All the work reported was conducted by myself in consultation with Dr. James O. Schenk, who shares authorship of the submitted manuscript as a second author.

The human embryonic kidney cells transfected with rat dopamine transporter were a gift from Dr. Marc Caron of Duke University. The human embryonic kidney cells transfected with the human dopamine transporter were a gift from Dr. Amy Eshleman of Oregon Health Sciences University.

Dedication

This thesis is dedicated to the memory of Paul Diedrich Schumacher for the bond we shared and who taught me that my life is what I make of it.

CHAPTER ONE

INTRODUCTION

One of the most important processes in biology is the transport of materials across cellular and intracellular membranes. Nutrients such as sugars and amino acids are needed within the cell and subsequent waste products within the cell need to be expelled. The lipid bilayer structure of membranes surrounding the cell creates problems with access into and out of the cell. Specialized transport mechanisms provide the solution to this problem by controlling what may cross membranes, thus regulating metabolism within the cell.

Transport across membranes can be generally characterized by three modes; simple, facilitated and active transport (Robinson, 1997). Simple transport is diffusion between two compartments that obeys Fick's laws in which the diffusing substrate distributes itself across the membrane until its chemical potential is equal on both sides of the membrane. Facilitated transport is a carrier mediated transport process that obeys Fick's laws and operates to dissipate electrochemical gradients. Active transport pumps materials across the membrane against a chemical potential gradient (Wilbrandt and Rosenberg, 1961). It accomplishes this task by utilizing a source of energy. In active transport there are two types of systems, primary and secondary, that are defined by the source of energy used (Mitchell, 1963). Primary active transport systems move material across the cell membrane by coupling movement with the energy released from ATP hydrolysis. An example of a primary active transport system is the Na^+/K^+-ATPase pump (Robinson, 1997). Secondary active transport systems couple the movement of

material across the membrane with the chemical energy available from asymmetric distribution of another ion across the membrane.

The concept of active transport coupled to an ion gradient can be traced back to 1900 when an excitation effect of NaCl was witnessed on the uptake of sugar by the small intestine (Reid, 1900, 1902). Riklis and Quastel (1958), witnessed that sugar accumulation ceased in the absence of Na^+ concluding that it was a required component. In 1960, R.K. Crane proposed that sugar adsorption in the intestine was directly coupled to the transport of Na^+ (Crane, 1960). This proposal was based upon five known facts regarding active sugar transport. These facts are 1) The process exhibited Michaelis-Menten kinetics; 2) it required energy; 3) it did not seem to include a chemical reaction; 4) free sugar accumulated within the cells and 5) the presence of Na^+ was required (Crane, 1977). Others, (Eddy, 1968; Schultz and Curran, 1970) have validated that at least part of the driving force for amino acid uptake is an inward electrochemical potential gradient of Na^+. So many mechanisms have now been based upon this principle of coupling organic solute transport to ion active transport that the concept has been identified as "The Na^+-Gradient Hypothesis" (Crane, 1977).

In order to validate the Na^+-gradient hypothesis in a given transport system one must show that Na^+ is accumulated in stoichiometric amounts along with the substrate (Jacquez, 1972). In general, there are four methods that have been devised for measuring the stoichiometry of coupled transport systems (Turner, 1985). Each of these methods have their own advantages and disadvantages that can preclude them from an experimental design, but are generally adaptable to almost any experimental design.

The first method is the direct method. This is the most straightforward method where measurements of the activator (ion) and substrate movement (flux) via the transporter are measured simultaneously. There are two ways to accomplish this measurement: 1) measure the total transporter related flux in the presence and absence of a specific transporter inhibitor or 2) measure the ion-dependent substrate flux and the substrate-dependent ion flux (Turner, 1985). A limitation of this method is that ion fluxes via the transporter may be small relative to background ion fluxes and thus difficult to measure. This technique has been successfully applied to Na^+-dependent glucose transport in brush border membranes (Turner and Moran, 1982).

The second method is the activation method where the substrate flux is measured as a function of ion concentration or ion flux as a function of substrate concentration. Analysis of results from this method could show a hyperbolic dependence of substrate flux to ion concentration or a sigmoidal dependence of substrate flux to ion concentration. A hyperbolic dependence is indicative of a 1:1 stoichiometry while a sigmoidal dependence indicates the involvement of multiple ions. The most common method of interpreting this data is based on analysis in terms of the Hill equation (Segel, 1993).

$$\nu = V_{max}\, A^n/(K' + A^n) \qquad \text{(Equation 1)}$$

The term ν and V_{max} are the net flux of substrate and maximal flux of the substrate respectively. A is the ion concentration, n is the coupling ratio or apparent stoichiometry and K' the equilibrium constant comprised of the coupling ratio and the substrate dissociation constant in the form of K^n_A. A major limitation to this method is that there is no distinction between "energetic" and "catalytic" coupling mechanisms (Turner and

Moran, 1982). The term energetic implies that the ion is cotransported with the substrate and catalytic implies that the ion is an essential activator but may not be transported. This limitation of the activation method renders it, by itself, a good indicator of stoichiometry but not necessarily conclusive evidence.

The third method used to determine ion-coupled substrate stoichiometry is the steady-state method. In this method, the steady-state substrate gradient is determined in the presence of a known ion chemical gradient (Lanyi, 1978; Johnson et al., 1981). If the transporter is operating at thermodynamic equilibrium and the membrane potentials are clamped at zero then the stoichiometry can be determined from the following equation

$$(S_i/S_o) = (A_o/A_i)^n \qquad \text{(Equation 2)}$$

where S denotes substrate concentration and A denotes ion concentration, n is the coupling ratio and the subscripts *i* and *o* denote intracellular and extracellular regions. Equation 2 is known as the fundamental rule of cotransport and shows that, at steady state, the concentration ratios of the driving ion and the driven substrate are inversely related (Stein, 1990; Weiss, 1996). There are three requirements for applying the steady-state method that are more strenuous than the other methods. The system must be capable of maintaining an ion gradient over time, leak pathways in the system must be small and contamination of the system must be minimal. Leak pathways or leakage is defined as any movement of the driven or driving substrate down its concentration gradient without the intervention of the transporter either internally or externally (Stein, 1986).

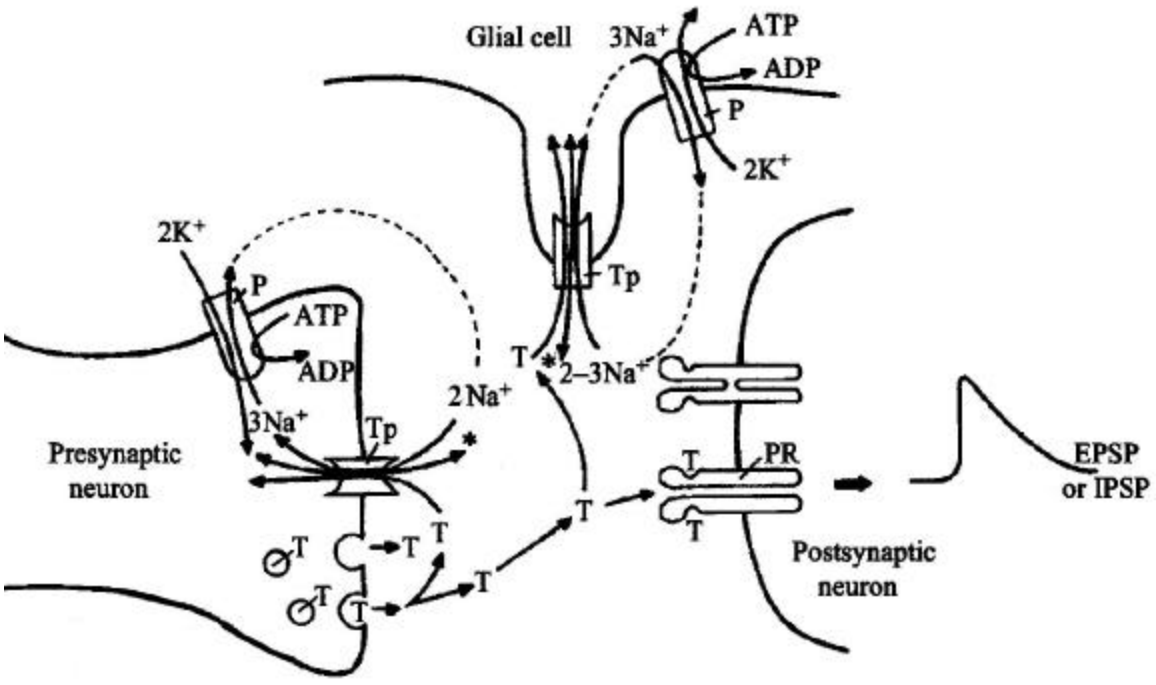
The fourth and final method for measuring ion-coupled substrate stoichiometry is known as the static head method. The static head method is similar to the steady state

method except that transport rates are used to determine experimental conditions where equation 2 holds true instead of steady-state distribution ratios (Turner and Moran, 1982). Both the steady-state method and the static head method are based on the argument of a tightly coupled transport mechanism. The term tightly coupled means that the translocation of substrate or ion via the transporter cannot occur in the absence of substrate or ion.

Na^+-coupled transport mechanisms are one of the most pervasive mechanisms by which solutes are moved across membranes against their electrochemical gradients (Wheeler, 1993). All levels of organisms from bacteria to mammals contain Na^+ coupled transport systems (Schultz and Curran, 1970). The diversity of purpose range from sugar and amino acid absorption through epithelial cell layers of the kidney (Turner and Moran, 1982) to concentrative uptake of neurotransmitters from the synaptic cleft (Kanner, 1983). Membrane transport systems are crucial to neuronal function and appear to be the primary mechanism which terminates the overall process of synaptic transmission (Fig. 1) (Iverson, 1975; Kuhar, 1973; Amara and Kuhar, 1993; Kanner, 1994). All neurotransmitters, γ-aminobutyric acid, L-glutamate, glycine, dopamine, serotonin and norepinephrine have their own high-affinity Na^+-dependent transporter (Kanner, 1994; Povlock et al., 1996). Of these neurotransmitters, serotonin, dopamine and norepinephrine are classified as biogenic amine neurotransmitters and belong to a second group of transporters that require not only Na^+ but also Cl^- for activation (Nelson, 1998). These simple molecules, serotonin, dopamine, and norepinephrine have highly complex actions in the peripheral and central nervous systems ranging from the control of

FIG. 1. Role of Neurotransmitter Transporter in Synaptic Transmission.

Neurotransmitter (T), stored in synaptic vesicles, is released by fusion of the vesicles with the synaptic plasma membrane. After its diffusion across the synaptic cleft, it binds to postsynaptic receptors (PR) resulting in excitatory or inhibitory postsynaptic potentials (EPSP or IPSP). Transmitter is removed from the cleft by re-uptake mediated by Na^+-coupled transporters (Tp) that also translocates other ions (*). In the case of transporters of dopamine, * is chloride and moves in the same direction as Na^+ and the neurotransmitter. The main driving force for this process is the electrochemical gradient of Na^+, which is maintained by the Na^+-pump (Na^+/K^+-ATPase, P). The transporters are located in the synaptic plasma membrane and also in the processes of glial cells, which are in close contact with the synapse. Figure adapted from Kanner (1994).


Glial cell
3Na+
ATP
ADP
P
2K+
2K+
P
ATP
ADP
3Na+
Tp
2Na+
Tp
T
2–3Na+
Presynaptic
neuron
T
PR
T
T
EPSP
or IPSP
Postsynaptic
neuron

heart rate to the fine-tuning of movement and the coloring of mood (Blakely and Bauman, 2000).

Dopamine and the neuronal dopamine transporter (DAT) have been studied since the 1960's. Dopamine is of interest because it has been linked to psychosis, drug abuse (Nestler, 1992; Carroll et al., 1999), Parkinson's disease (Hornykiewicz, 1979) and attention deficit hyperactivity disorder (Ernst et al., 1998). DAT is of particular interest because it is believed to be the site of action for drugs of abuse (Ritz et al., 1987; Bergman, et al., 1989; Spealman et al., 1989) and a site of entry for neurotoxins such as 1-methyl-4-phenylpyridinium ion (MPP^+) that can produce movement disorders similar to Parkinson's disease (Javitch et al., 1985).

Early studies of dopamine uptake in synaptosomes prepared from rat striatum by Harris and Baldessarini (1973) and Holz and Coyle (1974) showed a rapid, temperature-dependent process with characteristics of saturable kinetics. Both determined the optimal concentration for Na^+ was between 150 – 160 m*M*, a value in agreement with physiological extracellular levels. Their results also showed a direct dependence of dopamine uptake to extracellular Na^+. No mechanisms for substrate transport were predicted and no measurement of intracellular Na^+ accumulation was conducted. Kuhar and Zarbin (1978) tested for Cl^- dependence of dopamine transport in synaptosomes prepared from rat striatum. Their results showed conclusively a direct dependence of dopamine uptake on [Cl^-]. Again, no mechanisms were proposed and no intracellular measurements conducted.

Shank et al. (1987) studied the inhibitory effects of ion substitutes in neurotransmitter uptake in synaptosomes prepared from rat striatum. In regards to dopamine uptake, their results confirmed previous experiments and showed that with total removal of extracellular Na^+ or Cl^-, transport was totally blocked. Krueger (1990) conducted kinetic studies of dopamine uptake in synaptosomes prepared from rat striatum. The results of this work established that dopamine uptake had a second order dependence on extracellular Na^+ and a first order dependence on extracellular Cl^- suggesting two or more Na^+'s and one Cl^- were involved in transport. Also, results suggested that the transport process was electrogenic. As transport proceeded, the membrane potential changed from its resting potential. Interestingly, results of Krueger's experiments contrasted with results from Shank et al. (1987), in that when extracellular Na^+ was totally removed transport was only partially inhibited by 70-85%. Although Krueger determined an ordered dependence for Na^+ and Cl^-, based upon the method used, the results were too inconclusive to attempt to establish any type of quantitative model or definitely determine the stoichiometry.

McElvain and Schenk (1992) conducted kinetic studies in striatal suspensions and synaptosomes from rat striatum. The use of striatal suspensions allowed for kinetic resolution of dopamine uptake and afforded the opportunity to delineate the binding steps in uptake. As a result, the first multi-substrate kinetic mechanism was proposed in which results showed a random binding between one dopamine molecule and two Na^+ followed by binding of one Cl^-, which then initiated transport. In the model, two Na^+'s and one Cl^- were proposed from dependence measurements conducted in this work which agreed with Krueger (1990). Subsequently, Wheeler (1993) conducted uptake experiments with

synaptosomal preparations from rat striatum and then fit the results of the experiments to a computer model. The outcome was a validation of the model proposed by McElvain and Schenk along with validation of an electrogenic transport process. Povlock and Schenk (1997) conducted kinetic experiments with suspensions from the rat nucleus accumbens. Utilizing the same technique as McElvain and Schenk, the multisubstrate kinetic mechanism was determined to be identical to the mechanism proposed for dopamine transport in the striatum. However, the results also showed that dopamine transport was more tightly regulated in the nucleus accumbens with regard to Na^+. This was determined by analysis of the kinetic constants and showed that the nucleus accumbens possessed a greater degree of cooperativity between dopamine and Na^+.

Gu et al. (1994) conducted experiments with rat dopamine transporters (rDAT) stably expressed in a cell line that contained no native rDAT. This type of expression system provides a suitable model to study the activity and function of the transporter protein without native interference. Results of the study showed that rate of transport was a simple hyperbolic function of Cl^- and a sigmoidal function of Na^+. These functions are suggestive of one Cl^- and two or more Na^+ involved in the transport event. These results are in agreement with previous work conducted in native systems and further validates the hypothesis of two Na^+ and one Cl^- acting as cosubstrates during transport. Earles and Schenk (1999) conducted kinetic experiments with human dopamine transporter (hDAT) stably expressed in human embryonic kidney 293 (HEK 293) cells. The results showed a reaction order of two and one for Na^+ and Cl^- respectively. Also the multisubstrate kinetic mechanism was proposed in which the mechanism is ordered with two Na^+'s binding randomly followed by one dopamine molecule and then one Cl^- which

then initiates transport. The difference of ordered binding for hDAT in HEK 293 cells versus random binding in native systems could not be conclusively defined as an actual mechanistic difference between human and rat transporters or a function of the transporter protein expressed in a membrane with different functions from the native system.

Chen et al. (1999) conducted cationic substitution studies with hDAT stably expressed in HEK 293 cells. Results from this work were in agreement with Shank et al., (1987) in that different ion substitutes resulted in some degree of inhibitory effects on dopamine transport. These results brought into question the reaction order dependence determinations for Na^+ from all the previous reports owing to the fact that cationic substitutions are required to maintain constant osmolarity and ionic strength when the concentration of Na^+ is varied.

From the information cited above a general hypothesis can be formulated with the following points: 1) dopamine should stimulate the accumulation of Na^+, 2) accumulation of Na^+ should be tightly coupled with the transport of dopamine, 3) blocking dopamine transport with inhibitors should block Na^+ accumulation, and 4) the stoichiometry of Na^+ accumulation should be twice the amount of dopamine transported.

Subsequent discussion briefly reviews the background of the techniques employed in this study and places them in the context of techniques used by others. In the previous reports just reviewed, two techniques were predominately used for direct chemical measurements. The first technique was [^{3}H]Dopamine injected into synaptosomal preparations (Harris and Baldessarini, 1973; Holz and Coyle, 1974; Kuhar and Zarbin, 1978; Shank et al., 1987; Krueger, 1990; Wheeler et al., 1993) or expression

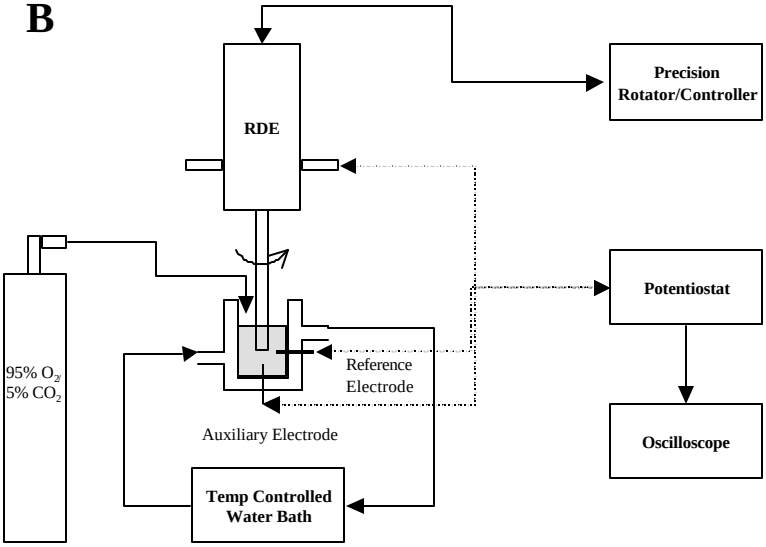
systems (Gu et al., 1994) and analyzed by liquid scintillation counting which has been the most widely used method for monitoring transport since the 1960's. The second technique was rotating disk electrode (RDE) voltammetry used with striatal suspensions (McElvain and Schenk, 1992; Povlock and Schenk, 1997) or expression systems (Chen et al., 1999; Earles and Schenk, 1999). This technique provides better resolution of transport because it allows for a continuous recording with excellent temporal resolution. The use of RDE voltammetry in analytical chemistry began in the 1960's after Levich solved the problem of quantifying diffusion at a RDE (Galus, 1976). In using RDE voltammetry, the working electrode is kept at a constant +450 millivolts versus a Ag/AgCl reference electrode. This potential is sufficient to oxidize dopamine when it is near the electrode surface to dopamine orthoquinone, which releases two electrons. The electrons are quantified at the RDE surface as current flow in a potentiostatic circuit (Earles et al., 1998).

A typical experimental setup includes a water-jacketed, temperature-controlled glass incubation chamber with the reference and auxiliary electrodes sealed in the chamber (Fig 2a). The chamber is perfused constantly with 95% O_2–5% CO_2 gas. The RDE is placed in the incubation chamber and corresponding working, reference and auxiliary leads are connected to a potentiostat that feeds into an oscilloscope for collection of the data. The RDE is precision rotated by a rotator/controller (Fig 2b). While a very effective technique, RDE voltammetry has two limitations that restrict its usefulness for stoichiometric coupling determinations. The first limitation is that the analyte of interest must be electroactive. The second limitation is that the

Fig 2. RDE Sampling Chamber and Diagram of RDE Experimental Setup. Panel A is a full-size photograph of the sampling chamber used in RDE voltammetry measurements of striatal or nucleus accumbens suspensions and DAT expressing HEK 293 cells. The attached wires are 0.5mm Pt that have been coated with Ag conducting paint and then anodized in a 0.1 M solution of HCl to produce Ag/AgCl reference and auxiliary electrodes. Panel B is a schematic diagram showing the setup as described in the text.

A

B


RDE
Precision Rotator/Controller
Potentiostat
Oscilloscope
95% O_2/ 5% CO_2
Reference Electrode
Auxiliary Electrode
Temp Controlled Water Bath

measurements can only be conducted extracellularly, which prohibits the direct measurement of intracellular mass accumulations.

A different technique applicable to quantitative determinations of stoichiometry with extreme sensitivity and excellent temporal and spatial resolution is fluorescence spectroscopy (Grinstein and Putnam, 1994). Fluorescence is a phenomenon where a molecule absorbs light, transiently stores the energy with electrons in an excited state and then emits light of lower energy. The intensity of the fluorescence emission can be calculated by the following equation:

$$I_F = I_o \gamma \varepsilon c l \qquad \text{(Equation 3)}$$

where I_F is the fluorescence intensity, I_o the intensity of incident light, c the concentration of fluorophore, γ the quantum yield, l the pathlength of the incident light in the sample and ε the molar absorptivity. The quantum yield and the molar absorptivity are two intrinsic properties of the fluorescent molecule. The molar absorptivity is a measure of the probability of absorption of incident light by the fluorophore. The quantum yield is defined as the number of quanta of light emitted relative to the number of quanta absorbed which is essentially a measure of the efficiency of the fluorescent process (Dissing and Gasbjerg, 1994).

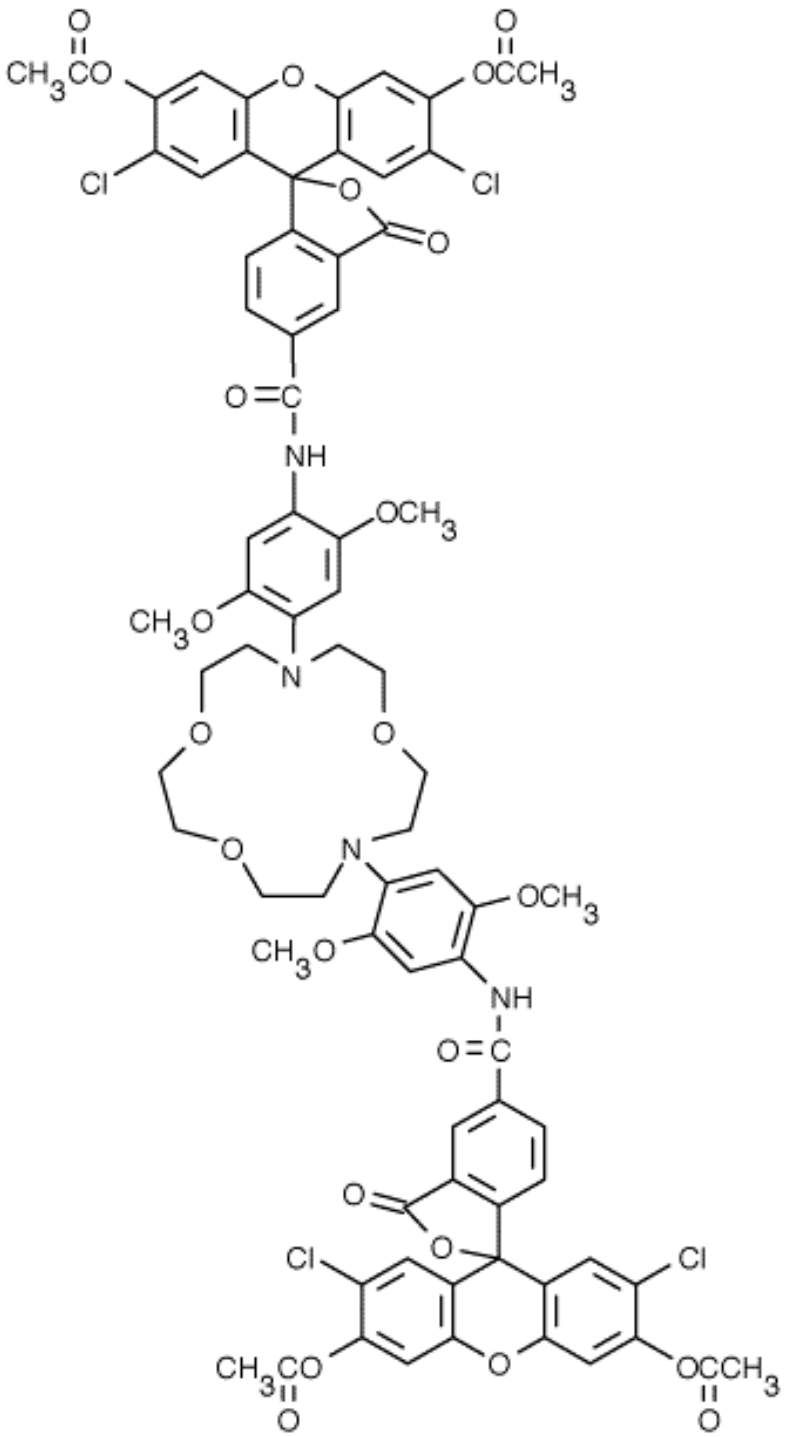
Most fluorescence measurements are conducted utilizing a fluorescent dye as a probe or as a label. The term probe refers to a dye that is environmentally sensitive and this sensitivity is used to monitor a particular parameter. The term label is used when a dye is employed as a marker that binds to a specific site or molecule. In either case, the ideal dye is one which observes an event but does not participate or interfere (Slavik, 1993). The great advantage of fluorescence as an optical technique is that it can be used

to investigate samples *in vivo* or *in vitro* under natural biological conditions. The measurement is nondestructive and almost noninvasive due to the low concentration of nontoxic dyes required.

Because all biological material exhibit some fluorescence, it is important to choose an applicable dye which is distinguishable from the background signal. Intrinsic or autofluorescence of biological materials is higher when ultraviolet excitation is used. For this reason, when working with biological samples a preferred dye would be one that is excitable in the visible spectrum (Slavik, 1993).

In recent years there has been rapid progress in the synthesis and use of fluorescent dyes that monitor intracellular ion concentrations. This was due to the availability of dyes as acetoxymethylesters (AM esters) which are membrane permeable (Haugland, 1996). The AM group masks the charge on the dye making it neutral and allows it to permeate through the membrane where nonspecific enzymic reactions, catalyzed mainly by esterases, cleaves off the AM group leaving the charged dye trapped within the membrane (Tsien, 1989). Currently there are three dyes available that are Na^+ sensitive; sodium-binding benzofuran isophtalate (SBFI), sodium-binding benzofuran oxazole (SBFO), and Sodium Green™. All three dyes have a crown ether ring which confers selectivity for Na^+ over K^+. The difference in the dyes is the attached fluorophore to the crown ether ring. SBFI and SBFO have almost identical benzofuran fluorophores with maximum excitation around 340nm. Sodium Green™ has fluorescein analogs attached to the crown ether ring (Fig. 3) with a maximum excitation at 507nm. Comparison of Sodium Green™ and SBFI shows that Sodium Green™ has higher

Fig. 3. Structure of Sodium Green™. Structure of Sodium Green™ tetraacetate in its cell-permeant form. The molecular formula is $C_{76}H_{64}Cl_4N_4O_{23}$ with a corresponding molecular weight of 1543.17 g/mol. In the form shown, Sodium Green™ will freely diffuse across the cell membrane. Once inside, the acetate moieties are cleaved producing a net negative charge that greatly reduces passive leakage from the cell. The molar absorptivity at pH 7.0, excitation 507nm, is 160,000 $cm^{-1}M^{-1}$. The K_d for Na^+ is 6 m*M*, 22°C, in the absence of K^+ and 21 m*M*, 22°C, in the presence of Na^+ and K^+, total ion concentration 135 m*M*. Figure adapted from Haugland (1996).



selectivity for Na^+ (41 fold vs. 18 fold) and a higher quantum yield (0.2 vs. 0.08) (Haugland, 1996). Given that Sodium Green™ is excited in the visible region, more selective and has a greater probability of emitting a photon it is the superior dye for a biological sample and was therefore the dye used in this study.

Another technique that is suitable for stoichiometric measurements is flame atomic emission spectroscopy, often referred to as flame photometry. Although flame photometry is not widely used for Na^+-coupled transport measurements, the technique is well suited for the determination of alkali metals such as Na and K. These metals are excited at relatively low temperatures and emit spectra which are simple and free from other metallic interferences. Most alkali metal spectra consist of relatively few, very intense lines that are well suited to quantitative emission measurements (Skoog, 1998). The appeal of this technique is that the instrumentation and sample preparation are relatively simple. Briefly, the sample to be analyzed is introduced into the instrument in an aqueous phase. The sample is nebulized into the gas flow which is pulled down a baffled tube and into the flame. The analyte is excited by the flame and emits light which is detected by a filter photometer and feed to a digital readout. The disadvantages of this technique are that the sample has to be in the aqueous phase and the sample size required is large because most of the sample is not nebulized and feeds into a waste container. The technique is destructive and the detection limit for alkali metals is on the order of 100 parts per billion, in the case of Na^+ approximately 4 μM (Skoog, 1998).

Although most of the studies of Na^+-coupled transport with biogenic amines have involved radiolabeled substrate or RDE voltammetry, previous investigations have been conducted using fluorescence and flame photometry to validate the Na^+-Gradient

Hypothesis using cytoplasts derived from Ehrlich ascites tumor cells (Henius and Laris, 1979).

Based on the hypothesis previously stated, the goal of this research is to show that by combining the techniques of RDE voltammetry and fluorescence utilizing the fluorescent dye Sodium Green™ in HEK 293 cells expressing DAT: 1) intracellular concentrations of Na^+ can be measured simultaneously with dopamine transport; 2) dopamine stimulates transport of Na^+ and both are tightly coupled to the transporter; 3) intracellular Na^+ accumulation and dopamine transport are blocked by inhibitors of DAT activity; 4) the mass accumulation of Na^+ is twice the accumulation of dopamine; and 5) flame photometry will be used as a confirmation technique.

REFERENCES

Amara S. G. and Kuhar M. J. (1993) Neurotransmitter transporters: recent progress. *Ann. Rev. Neurosci.* **16**, 73-93.

Bergman J., Madras B. K., Johnson S. E. and Spealman R. D. (1989) Effects of cocaine and related drugs in nonhuman primates III. Self-administration by squirrel monkeys. *J Pharmacol. Exp. Ther.* **251**, 150-155.

Carroll F. I., Howell L. L. and Kuhar M. J. (1999) Pharmacotherapies for treatment of cocaine abuse: Preclinical aspects. *J. Med. Chem.* **42**, 2721-2736.

Chen N., Trowbridge C.G., and Justice J.B. (1999) Cationic modulation of human dopamine transporter: dopamine uptake and inhibition of dopamine uptake. *J. Pharmacol. Exp. Ther*. **290**, 940-949.

Crane R. K. (1960) Intestinal absorption of sugars. *Physiol. Rev*. **40**, 789-825.

Crane R. K. (1977) The gradient hypothesis and other models of carrier mediated active transport. *Rev. Physiol. Biochem. Pharmacol*. **78**, 99-159.

Dissing S. and Gasbjerg P. K. (1994) Basic principles behind the application of fluorescent dyes in *Methods in Membrane and Transporter Research* (Schafer J. A., Giebish G., Kristensen P. and Ussing H. H. eds), pp. 143-176. R. G. Landes Co., Austin, TX.

Earles C. and Schenk J. O. (1999) Multisubstrate mechanism for the inward transport of dopamine by the human dopamine transporter expressed in HEK cells and it inhibition by cocaine. *Synapse* **33**, 230-238.

Earles C., Wayment H., Green M., and Schenk J. O. (1998) Resolution of biogenic amine transporter kinetics by rotating disk electrode voltammetry: methodology and mechanistic interpretations. *Meth Enzymol* **296**, 660-675.

Eddy A. A. (1968) The effects of varying the cellular and extracellular concentrations of sodium and potassium ions on the uptake of glycine by mouse ascites-tumor cells in the presence and absence of sodium cyanide. *Biochem J.* **108**, 489-498.

Ernst M., Zametkin A. J., Matochik J. A., Jons P. H. and Cohen R. M. (1998) DOPA decarboxylase activity in attention deficit hyperactivity disorder adults. A [fluorine-18] fluorodopa positron emission tomographic study. *J. Neurosci.* **18**, 5901-5907.

Galus Z. (1976) *Fundamentals of Electrochemical Analysis*, pp. 38-43. John Wiley and Sons Inc.: New York.

Grinstein S. and Putnam R. (1994) Measurement of intracellular pH in *Methods in Membrane and Transporter Research* (Schafer J. A., Giebish G., Kristensen P. and Ussing H. H. eds), pp. 113-141. R. G. Landes Co., Austin, TX.

Gu H., Wall S.G., and Rudnick G. (1994) Stable expression of biogenic amine transporters reveals differences in inhibitor sensitivity, kinetics, and ion dependence. *J. Biol. Chem*. **269**, 7124-7130.

Harris J. F. and Baldessarini R. J. (1972) The uptake of [^{3}H]dopamine by homogenates of rat corpus striatum: effects of cations. *Life Sci* **13**, 303-313.

Haugland R. P. (1996) *Handbook of Fluorescent Probes and Research Chemical: Sixth Edition.* (Spence M. T. Z. ed), pp 503-580. Molecular Probes Inc, Eugene Oregon.

Henius G. V. and Laris P. C. (1979) The Na^+ gradient hypothesis in cytoplasts derived from Ehrlich ascites tumor cells. *Biochim. Biophys. Res. Commun*. **91**, 1430-1436.

Holz K. W. and Coyle J. T. (1974) The effects of various salts, temperature, and alkaloids, veratridine and batrachotoxin on the uptake of [^{3}H]-dopamine into synaptosomes from rat striatum. *Mol. Pharm*. **10**, 746-758.

Hornykiewicz O. (1979) Brain dopamine in Parkinson's disease and other neurological disturbances, in The Neurobiology of Dopamine (Horn A.S., Korf J. and Westerink B.H.C., eds), pp.633-654. Academic Press: New York.

Iverson L. L. ed (1975) *Handbook of Psychopharmacology, Vol 2*. pp. 381-442. Plenum Press, New York.

Javitch J. A., D'Amato R. J., Strittmatter S. M. and Synder S. H. (1985) Parkinsonism-inducing neurotoxin, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: Uptake of the metabolite *N*-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity. *Proc. Natl. Acad. Sci. USA* **82**, 2173-2177.

Jacquez J. A. (1972) Ion gradient hypotheses and the energy requirement for active transport of amino acids. In: Na-linked transport of organic solutes: the coupling between electrolyte and no electrolyte transport in cells (Heinz E, ed), pp 4-14 Berlin: Springer-Verlag.

Johnson R. G., Carty S. E. and Scarpa A., (1981) Proton: substrate stoichiometries during active transport of biogenic amines in chromaffin ghosts. *J. Biol Chem.* **256**, 5773-5780.

Kanner B. I. (1983) Bioenergetics of neurotransmitter transport. *Biochim. Biophys. Acta* **726**, 293-316.

Kanner B. I. (1994) Sodium-coupled neurotransmitter transport: structure, function and regulation *J. Exp Biol.* **196**, 237-249.

Krueger B. K. (1990) Kinetics and block of dopamine uptake in synaptosomes from rat caudate nucleus. *J. Neurochem*. **55**, 260-267

Kuhar M. J. (1973) Amino acid transport: alterations due to synaptosomal depolarization. *Life Sci.* **13**, 1623-1634.

Kuhar M. J. and Zarbin M. A. (1978) Synaptosomal transport: a chloride dependence for choline, GABA, glycine, and several other compounds. *J. Neurochem*. **31**, 251-256.

Lanyi J. K. (1978) Coupling of aspartate and serine transport to the transmembrane electrochemical gradient for sodium ions in *Halobacterium halobium*. Translocation stoichiometries and apparent cooperativity. *Biochemistry* **17**, 3011-3018.

McElvain J. S. and Schenk J. O. (1992) A multisubstrate mechanism for uptake of striatal dopamine uptake and its inhibition by cocaine. *Biochem. Pharmacol*. **43**, 2189-2199.

Mitchell P. (1963) Molecule, group and electron translocation through natural membranes. *Biochem Soc. Symp.* **22**, 142-169.

Nelson N. (1998) The family of Na^+/Cl^- neurotransmitter transporters *J. Neurochem.* **71**, 1785-1803.

Nestler E. J. (1992) Molecular mechanisms of drug addiction. *J. Neurosci.* **12**, 2439-2450.

Povlock S. L. and Schenk J. O. (1997) A multisubstrate kinetic mechanism of dopamine transport in the nucleus accumbens and its inhibition by cocaine. *J. Neurochem*. **69**, 1093-1105.

Reid E. W. (1900) On intestinal absorption, especially on the absorption of serum, peptone and glucose. *Phil. Trans. B*. 192-211.

Reid E. W. (1902) Intestinal absorption of solutions. *J Physiol. Lond.* **28**, 241-256.

Riklis E. and Quastel J. H. (1958) Effects of cations on sugar absorption by isolated surviving guinea pig intestine. *Can J. Biochem. Physiol.* **36**, 347-362.

Ritz M. C., Lamb R. J., Goldberg S. R. and Kuhar M. J. (1987) Cocaine receptors on dopamine transporters are related to self-administration of cocaine. *Science* **237**, 1219-1223.

Robinson J. D., ed (1997) *Moving Questions: A History of Membrane Transport and Bioenergetics.* pp 220-233 New York: Oxford University Press

Schultz S. G. and Curran P. F. (1970) Coupled transport of sodium and organic solutes. *Physiol Rev.* **50**, 637-718.

Segel I. H., ed (1993) *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems.* pp 340-385 New York: John Wiley & Sons, Inc.

Shank R. P., Schneider C. R., and Tighe J. J. (1987) Ion dependence of neurotransmitter uptake : Inhibitory effects of ion substitutes. *J. Neurochem.* **49**, 381-388.

Skoog D. A., Holler F. J. and Nieman T. A. eds (1998) *Principles of Instrumental Analysis, Fifth Edition.* pp. 206-251. Harcourt Brace and Co., Philadelphia, Pennsylvania.

Slavik J. ed (1994) *Fluorescent Probes in Cellular and Molecular Biology.* pp 2-231. CRC Press, London.

Spealman R. D., Madras B. K. and Bergman J. (1989) Effects of cocaine and related drugs in nonhuman primates. II. Stimulant effects on scheduled-controlled behavior. *J. Pharmacol. Exp. Ther*. **261**, 142-149.

Stein W. D. (1986) *Transport and Diffusion across Cell Membranes*. San Diego, CA: Academic Press, Harcourt Brace Jovanovitch, Publishers.

Stein W. D. (1990) *Channels, Carriers, and Pumps: An Introduction to Membrane Transport*. pp 127-171. San Diego, CA: Academic Press, Harcourt Brace Jovanovitch, Publishers.

Tsien R. Y. (1989) Fluorescent indicators of ion concentrations in *Fluorescent Microscopy of Living Cells in Culture.* (Wang Y. L. and Taylor D. L., eds), pp127-156. Academic Press Inc., New York.

Turner R. J. and Moran A. (1982) Stoichiometric studies of the renal outer cortical brush border membrane D-glucose transporter. *J. Memb. Biol*. **67**, 73-80.

Turner R. J. (1985) Stoichiometry of cotransport systems in *Annals of the New York Academy of Sciences Vol 456: Membrane Transport Driven by Ion Gradients* (Semenza G. and Kinne R., eds), pp 10-25. New York Academy of Sciences, NewYork.

Weiss T. F. (1996). Carrier-mediated transport. In: *Cellular Biophysics, Volume 1: Transport*. pp 333-447 Cambridge, MA: A Bradford Book, The MIT Press.

Wheeler D. D., Edward A. M., Chapman B. M., and Ondo J. G. (1993) A model of the sodium-dependence of dopamine uptake in rat striatal synaptosomes. *Neurochem. Res*. **18**, 927-936.

Wilbrandt W. and Rosenburg T. (1961) The concept of carrier transport and its corollaries in pharmacology. *Pharmacol. Rev*. **13**, 109-183.

CHAPTER TWO

AN INVESTIGATION OF THE STOICHIOMETRY OF Na^+ COTRANSPORT WITH DOPAMINE IN RAT AND HUMAN DOPAMINE TRANSPORTERS EXPRESSED IN HUMAN EMBRYONIC KIDNEY CELLS

ABSTRACT

The neuronal membrane transporter for dopamine (DAT) concentrates dopamine intracellularly up to 10^6 fold over extracellular levels, and Na^+ is thought to provide the major driving force. Results herein show in human embryonic kidney (HEK) cells expressing the rat DAT and HEK cells expressing the human DAT that: 1) dopamine stimulates transport of Na^+ as measured by flame photometry and the fluorescent sodium indicator, Sodium Green™; 2) the time course of accumulation of intracellular Na^+ is time-locked with dopamine; 3) the amount of Na^+ accumulated intracellularly is dependent on the extracellular concentration of dopamine; 4) dopamine-stimulated accumulation of Na^+ is blocked by both cocaine and mazindol; and 5) the mass of Na^+ accumulated when related to dopamine was found to be 2.6 ± 0.2 SD and 2.5 ± 0.2 SD for rat DAT and human DAT, respectively, when estimated by fluorescence spectroscopy and 1.9 ± 0.1 and 1.9 ± 0.2 for rat DAT and human DAT, respectively, when estimated by flame photometry. Taken together these results suggest that the stoichiometric coefficient for Na^+ transport with dopamine is around 2.

Key Words: flame photometry; fluorometric dyes; HEK-293 cells; rotating disk electrode voltammetry; Na^+-dependent transporters; Sodium Green

Running Title: Na^+ Stoichiometry at the Dopamine Transporter

Abbreviations used:

AT, auto-titrator; AUX, auxiliary electrode; CMF-PBS, calcium and magnesium free phosphate buffered saline; Coc, cocaine; CR, chart recorder; DAT, dopamine transporter; Ex, Excitation path; F, relative fluorescence; hDAT, human dopamine transporter; HEK, human embryonic kidney cells; Maz, mazindol; MC, monochromator; PMT, photomultiplier tube; POT, potentiostat; rDAT, rat dopamine transporter; RDE, rotating disk electrode; REF, reference electrode; SC, signal converter; SER, standard error of regression; Xe, Xenon short arc lamp

The neuronal transporter for dopamine (DAT) is a member of the Na^+ and Cl^- dependent family of transporters (Amara and Kuhar, 1993). DAT functions to clear extracellular dopamine during chemical neurotransmission. The results of early studies of DAT function suggested that it is a transporter which concentrates dopamine against its concentration gradient (Holz and Coyle, 1974) and the results of recent studies in native (Krueger, 1990; McElvain and Schenk, 1992; Wheeler et al, 1993; 1994; Povlock and Schenk, 1997) and expression systems (Earles and Schenk, 1999) suggest that the DAT can concentrate dopamine to intracellular values of 10^6 to 10^7 over extracellular values. Crane (1960; 1977) proposed that substrate accumulation is coupled to the transmembrane gradient of Na^+ in Na^+-dependent transporters and that the resulting energy can account for the concentrative property of this family of transporters. One of the important phenomena that must be proved in order to validate the Na^+-gradient hypothesis in a given transport system is to show that Na^+ is accumulated in stoichiometric amounts along with the substrate (Jacquez, 1972). Reviews of stoichiometric considerations of ion coupling in transporter function have appeared recently (Krupka, 1993; 1998). Stoichiometric relationships between substrate and Na^+ accumulation have been shown for a number of transporters (Stein, 1986; 1990; Weiss, 1996) and the energy requirements related to concentrative transport have been considered (Schafer, 1972). For example, the glucose transporter has been shown to transport glucose with one or two Na^+ depending on the location of the transporter protein (Turner and Moran, 1982). In direct chemical assays of DAT function it has been found that dopamine transport has a second order dependence on $[Na^+]_o$ in native (Bogdandski, 1976; Krueger, 1990; McElvain and Schenk, 1992; Wheeler et al., 1993;

1994; Povlock and Schenk, 1997) and in expression systems expressing the human DAT (hDAT) (Gu et al., 1994; Earles and Schenk, 1999). The results of electrophysiological studies in cells expressing the DAT suggest that the stoichiometry between dopamine and Na^+ (or other cation) can range from 1:2 to much higher values under some conditions (Sonders et al., 1997; Sitte et al., 1998; Sonders and Amara, 1998). How the ion coupling is directly related to the movement of dopamine itself is unknown in the cited electrophysiological studies because direct chemical measurements of dopamine and Na^+ were not made. The results of a recent report (Chen et al., 1999) suggest that the apparent coupling stoichiometry from assessment of reaction order may be a formal phenomena based on which ion substitute for Na^+ is used in a particular study. A hypothesis based on the information cited above is that: 1) extracellular dopamine should stimulate the accumulation of Na^+, 2) accumulation of Na^+ should be time-locked to the transport of dopamine, 3) inhibitors of DAT activity should block both dopamine transport and Na^+ accumulation, and 4) the stoichiometry of dopamine transport and Na^+ accumulation should be integerly related. Herein we show that intracellular concentrations of Na^+, $[Na^+]_i$, can be measured concomitantly with dopamine transport by combining rotating disk electrode (RDE) voltammetry with fluorometric monitoring of $[Na^+]_i$ in human embryonic kidney cells (HEK) expressing DAT using the commercially available, fluorescein labeled, crown ether indicator for Na^+, Sodium Green™. Results were confirmed by flame photometry and show that the stoichiometry is 2:1, Na^+ to dopamine. A preliminary presentation of some of this data was made at the 2000 meeting of the Society for Neuroscience (Schumacher et al., 2000).

MATERIALS AND METHODS

Fluorescence and Electroanalytical Setup

Fluorescence experiments were performed utilizing a modular design, dual-channel emission fluorimeter with a Xenon short arc lamp (Photon Technology International, PTI, Monmouth Junction, NJ U.S.A.) equipped with dual photomultiplier tubes (Type R1527P, No. MF6843, Hamamatsu Photonics K. K., Japan). For making fluorometric and electroanalytical chemical measurements, the following modifications were made to the fluorimeter (see Fig. 1). A hole was cut through and a support rod mounted onto the sample compartment lid that allowed the placement of a glassy carbon RDE (Pine Instruments, Grove City, PA U.S.A.) into the sample chamber without blocking or interfering with the light paths. The incubation chambers were polished quartz cuvettes. They were externally square (1 cm X 1 cm), custom-made by Spectrocell Inc (Oreland, PA U.S.A.), and had an internal cylindrical diameter of 8 mm. This design allowed for rapid convection of the contents of the cuvette by the RDE.

Electroanalytical chemical measurements were conducted as previously described (Meiergerd and Schenk, 1995; Earles et al., 1998; Earles and Schenk, 1998; 1999). In brief, the potential was applied and the signal current measured by a battery-powered potentiostat (Model LC-3D Petite Ampère from Bioanalytical Systems, West Lafayette, IN U.S.A.). The RDE was rotated at 2000 rpm with a Pine Instruments MSRX Precision Speed Controller. Auxiliary and reference electrodes, Ag and Ag/AgCl, respectively, were glued with Torr Seal (Varian Vacuum Products, Lexington, MA U.S.A.) to the external walls of the cuvette with the required electrical leads running through access ports in the back of the sample compartment of the spectrometer. The cuvette was aerated

constantly with 95% O_2 - 5% CO_2. Current output was recorded on a Bioanalytical Systems Inc. (Model RYYT) strip chart recorder. Dopamine injections were made with an auto-titrator, Micro-Lab® M (Hamilton, Inc., Reno, NV U.S.A.), through access ports on the back of the fluorescence sample compartment of the spectrometer. Data acquisition, storage, and analyses were performed using the software, FeliX™, Version 1.21, provided by PTI with subsequent data analysis utilizing Microsoft® Excel, Version 97 (Microsoft, Redmond, WA U.S.A.) and GraphPad Prism®, Version 3.0 (GraphPad Software, San Diego, CA U.S.A.).

Cell Preparation, Loading with Sodium Green™, General Measurement Protocol, and Determination of Intracellular Na^+ and Dopamine Uptake

HEK 293 cells, line 1 expressing hDAT and line 6 expressing rDAT, were gifts from Drs. Amy Eshlemann, Aaron Janowsky and Kim Neve at the VA Hospital at the Oregon Health Sciences University (Portland, OR U.S.A.) and M. Caron at Duke University (Durham, NC U.S.A.), respectively. The cells were cultured in the laboratory as previously described (Earles and Schenk, 1999). Briefly, cells were grown until the culture plates (100 mm diameter) were 90-95% confluent. To prepare for loading with Sodium Green™, the cells were rinsed twice with an excess of Ca^{2+} and Mg^{2+} free, pH 7.3, phosphate buffered saline (CMF-PBS, composition: 138 m*M* NaCl, 4.1 m*M* KCl, 5.1 m*M* Na_2HPO_4, 5.0 m*M* KH_2PO_4 and 2% (wt/vol) glucose) in the culture plate.

Sodium Green™ was loaded into the cells in a similar manner to that described by Amorino and Fox (1995) with minor modifications. In brief, 6 mL of pH 7.4 physiological buffer (composition: 124 m*M* NaCl, 1.80 m*M* KCl, 1.30 m*M* $MgSO_4$, 1.24

m*M* KH_2PO_4, 2.50 m*M* $CaCl_2$, 26.0 m*M* $NaHCO_3$ and 10.0 m*M* glucose saturated with 95% O_2 – 5% CO_2 gas) containing 2 μM Sodium Green™ tetraacetate, cell-permeant (Molecular Probes, Inc., Eugene, OR U.S.A.) was applied to the rinsed culture plate. Stock solutions of the dye consisted of 2 m*M* Sodium Green™ tetraacetate dissolved in equal volumes of anhydrous dimethylsulfoxide (Sigma Chemical Co., St. Louis, MO U.S.A.) and 25% (wt/vol) Pluronic F127 (Molecular Probes, Inc.) to improve loading of the acetoxymethyl ester form of Sodium Green™ (Amorino and Fox, 1995). Culture plates were incubated with the dye-containing buffer for 45 min at 37 ^oC then rinsed twice with CMF-PBS to remove any impermeant dye. Sodium Green™ is assumed to remain in the intracellular compartment because of the action of cellular esterases to produce an anion (carboxylate) form of the dye (Amorino and Fox, 1995; Friedman and Haddad, 1994; Harootunian et al., 1989). The cells were then detached from the culture plate by agitation with a stream of physiological buffer expelled from a pipette (2 mL buffer/100 mm culture plate). The cells were allowed to equilibrate for 30 min in a 37 ^oC water bath.

Aliquots of cells (150 μL) were then removed and added to 1 mL of physiological buffer in the quartz cuvettes for fluorescent and electrochemical analyses. All analyses were conducted with the RDE rotating the suspension of cells in buffer at 2000 rpm and aerated with the 95% O_2 – 5% CO_2 gas mixture. In some experiments physiological buffer containing 0 m*M* K^+ (instead of the 3.04 m*M*) was used to inhibit the Na^+/K^+ ATPase by not providing extracellular substrate for the enzyme. This condition has been shown to prohibit the activity of the Na^+/K^+ ATPase in a variety of cells and tissues (Stein, 1986; Garrahan and Glyn, 1967; Steinbach, 1954; Hodgkin and Keynes, 1954).

Sodium Green™ was excited at 507 nm and emission was measured at 532 nm and subsequently corrected for background and native fluorescence (details below). Na^+-dopamine uptake experiments were performed by the addition of varying concentrations of dopamine to the sample and monitoring the fluorescent intensity increase at 532 nm for Na^+ uptake and simultaneously monitoring the electrochemical signal decay for dopamine transport. After an analysis, the sample was quantitatively transferred to a microcentrifuge tube, sonicated, and a Bradford assay (Bradford, 1976) was performed to determine the total protein in the sample.

The background plus native fluorescence signals in the absence of Sodium Green™ were run in triplicate prior to each experiment and were transferred to Microsoft® Excel, averaged, and stored. Then the fluorescence signals obtained for each experiment with Sodium Green™ plus various extracellular concentrations of dopamine, $[DA]_o$, were measured, transferred to the Excel spread sheet and corrected for background and native fluorescence by subtraction. The results were normalized for the number of cells within the sample. The protein content (in μg) of the cell sample was estimated from the Bradford assay. The protein values were correlated to the number of cells present (measured by cell counting with a hemocytometer) by a calibration curve which was found to be linear with a conversion factor of 767 cells/μg protein. The total intracellular volume was estimated from the number of cells present and a microscopically measured diameter of 15 μm for HEK cells. The corrected spectrum was normalized for volume of cells defined by the number of cells and the calibration curve for Sodium Green™ fluorescence applied. This sequence provided estimates of the $[Na^+]_i$. The raw electrochemical signal of $[DA]_o$ versus time was analyzed in a similar

manner with the following exceptions. The signal was corrected for any baseline drift using a calibration curve. Where needed the $[DA]_i$ was estimated by measuring the number of moles of dopamine taken up divided by the intracellular volume. The ratio was then calculated by evaluating the ratio,$[Na^+]_i/[DA]_i$.

Calibration of $[Na^+]_i$

Calibration of fluorescence intensity in terms of $[Na^+]_i$ was accomplished *in situ* in separate experiments by application of gramicidin (Molecular Probes, Inc.). Gramicidin produces transmembrane pores that are highly selective for monovalent cations (Hille, 1992). Exposure of the cells to various concentrations of Na^+ in the presence of gramicidin permits calibration of fluorescence intensity in terms of $[Na^+]_i$. Cells were loaded with Sodium Green™ as described above and incubated in varying values of $[Na^+]$. Na^+ was substituted with N-methyl-d-glucamine hydrochloride (Tokyo Chemical Co., Tokyo, Japan) and choline bicarbonate (Aldrich Chemical Co., Milwaukee, WI U.S.A.) to maintain constant ionic strength. Cells were made permeable to Na^+ by the addition of 10 μ*M* gramicidin to 150 μL of cells in 1mL of Na^+ substituted physiological buffer. The cells were allowed to equilibrate for 10 min and fluorescent intensity recorded for 120 secs. The 10 min equilibration time was determined experimentally by addition of gramicidin and monitoring fluorescence at 532 nm for 20 min (data not shown).

Observed values of relative fluorescence, *F*, at three different known $[Na^+]_i$ values were substituted into the expressions given by Harootunian et. al (1989) for estimating the curve fitting parameters, F_{min} and F_{max}. The K_D was then estimated by non-linear

curve fitting using all of the values and the expression from Grynkiewicz et. al (1985), as given in the legend of Fig. 3 where the results of the calibrations are shown.

Flame Photometry Calibration and Analysis.

Independent estimations of the mass of Na^+ taken up with dopamine were made in separate experiments by flame photometry (Digital Flame Analyzer, Model 2655-00 with linearizer from Cole-Parmer Instrument Co., Chicago, IL U.S.A.). Flame photometry has been used successfully in studies of Na^+ cotransport by the glucose transporter (Henius and Laris, 1979) and in studies of Na^+/K^+ ATPases in HEK 293 cells (Grishin and Caplan, 1998). In the study here, 99.5% pure propane was used as the fuel with no scrubbing. Calibration of flame emission intensity as a function of $[Na^+]$ was made using standards made up in water from a Nanopure water purification system (Barnstead, Dubuque, IA U.S.A.). The experimentally determined detection limit at 2 times the signal to noise ratio was 0.7 μM Na^+. At the dilution used for the analyses performed the values of the $[Na^+]$ was in the range 3 μM to 5 μM.

Cell suspensions were prepared as previously described (Earles and Schenk, 1999) with exceptions. Briefly, culture plates (90 – 95% confluent) were washed twice with CMF-PBS and then the cells were detached from the plate with physiological buffer (2 mL per plate). Cells were spun in a desktop centrifuge, Eppendorf model 5403, (Brinkmann Instruments, Inc., Westbury, NY U.S.A.) at $30 \times g$ for 3 min. Supernatant was removed and cells were resuspended in physiological buffer (2 mL per plate) and allowed to equilibrate for 30 min. A 600 µL aliquot of cells in physiological buffer was injected with 5 μM dopamine and incubated at 37 ^{o}C for 15 min with gentle agitation if

the cells were visually observed to settle. Samples were then centrifuged at $121 \times g$ for 3 minutes and the supernatant removed. The cell pellet was resuspended in 5 mL of the Nanopure H_2O and sonicated. Samples were then analyzed for total [Na^+] by flame photometry. After analysis, 20 μL of the sample was removed for protein analysis using the Bradford assay. To inhibit transport, 100 μM of cocaine (final concentration) was added 30 seconds prior to the injection of dopamine (Earles and Schenk, 1999). The samples were then analyzed in the same manner as described above.

RESULTS

Intracellular Na^+ Can be Measured Fluorometrically in HEK Cells with Sodium Green™

Figure 1 is a schematic diagram of the instrumentation used to simultaneously measure dopamine transport and intracellular accumulation of Na^+. Practical details of the instrumental arrangement are given above in the Materials and Methods section. It was necessary to add the RDE to the existing spectrometer in order to correlate the accumulation of Na^+ with the transport of dopamine. The RDE served to monitor dopamine transport voltammetrically and concomitantly stirred the contents of the cuvette to ensure suspension of the cells in physiological buffer. The presence of the electrodes caused a slight increase in apparent fluorescence due to light scatter but was constant throughout all experiments and subtracted out as part of the background.

Figure 2A shows typical spectra observed during experimentation. These spectral features are those expected for the loading of Sodium Green™ into cells containing $[Na^+]_i$ (information supplied by the manufacturer, Molecular Probes, Inc, and others, Friedman and Haddad, 1994; Amorino and Fox, 1995; Szmacinski and Lakowicz, 1997). Figure 2B shows the time course of Na^+ binding to the Sodium Green™ following the addition of the pore-forming antibiotic, gramicidin, to a buffer of known $[Na^+]_o$ containing HEK293 rDAT cells. Apparent equilibrium is obtained in 10 min. Thus, further calibration procedures were conducted after incubation periods of 10 min with gramicidin. Although the exact timing of the emission signal relative to the binding of Na^+ to Sodium Green™ can not be measured in the intracellular compartment of HEK 293 cells, there is evidence indicating that the binding/emission phenomenon is rapid.

FIG 1. Schematic Diagram of Spectro-Electroanalytical Setup. Schematic diagram (viewed from above) of the spectro-electroanalytical setup for measuring the transport of dopamine and uptake of Na^+. Boxed Insert is an expanded schematic diagram (viewed from side) of the cuvette configuration used for measuring the transport of dopamine and Na^+. Note that the cuvette is rotated relative to the view in Panel A and the emission signals are normal to the plane of the page. Key: AT, auto-titrator; AUX, auxiliary electrode; CR, chart recorder; Ex, Excitation path; F, fluorescence emission path; MC, monochromator; PMT, photomultiplier tubes; POT, potentiostat; RDE, rotating disk electrode; REF, reference electrode; SC, signal converter; Xe, Xenon short arc lamp.

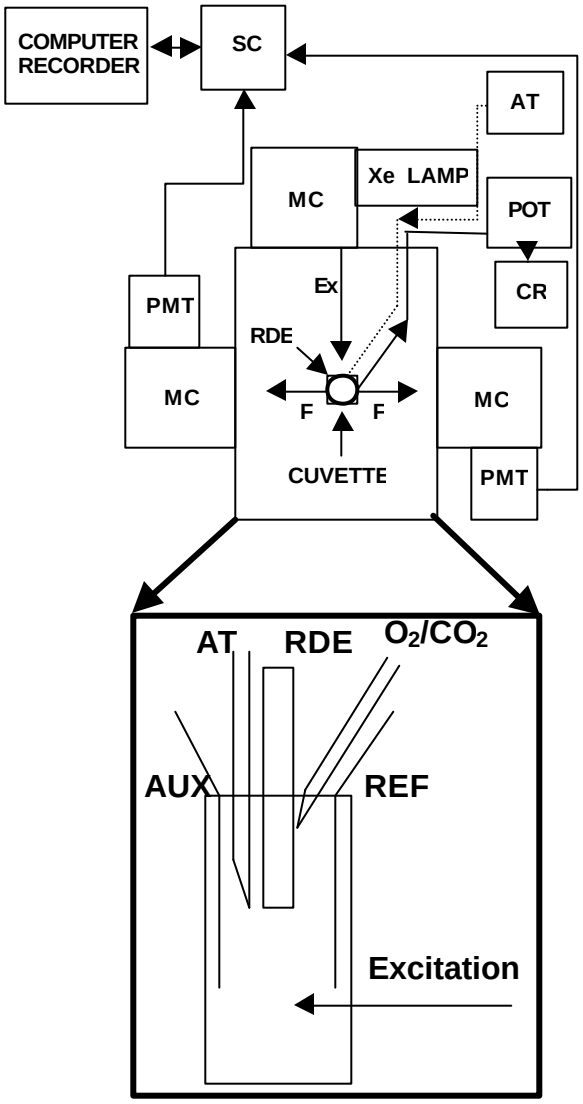
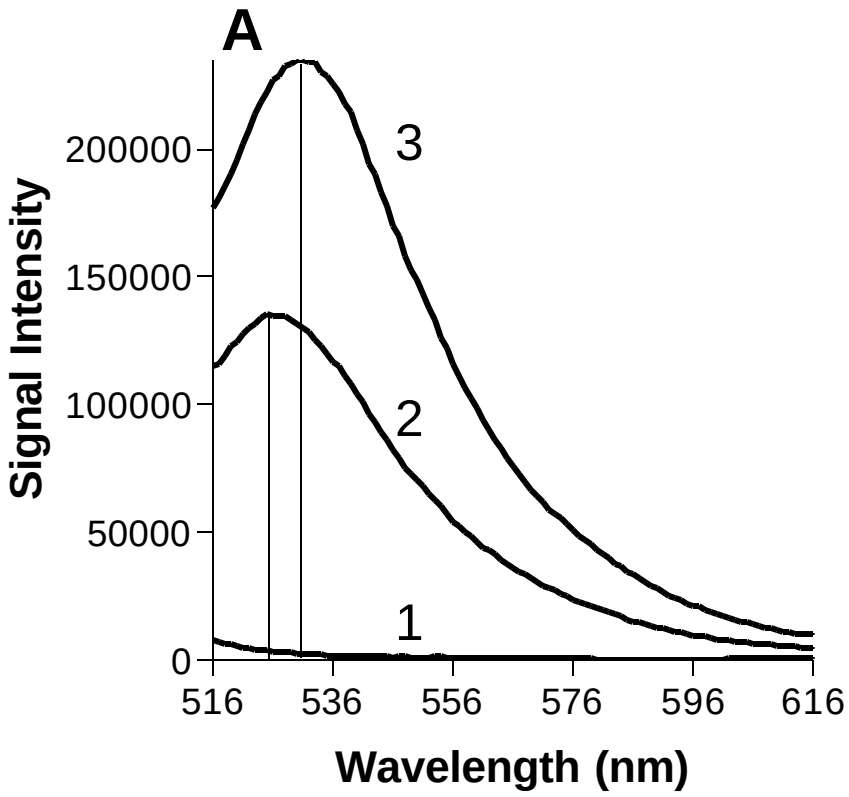
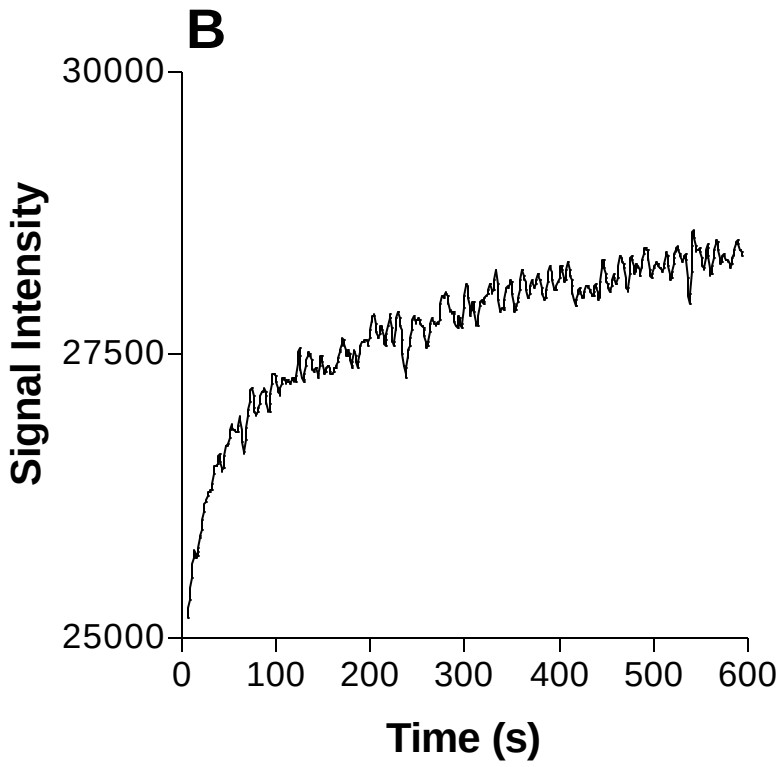

COMPUTER
RECORDER
SC
AT
MC
Xe LAMP
POT
CR
PMT
Ex
RDE
MC
MC
F
F
CUVETTE
PMT
AT
RDE
O_2/CO_2
AUX
REF
Excitation

FIG. 2. Spectral properties of Sodium Green™. Panel A shows three emission spectra (excitation wavelength at 507 nm), illustrating the spectral properties of Sodium Green™ within suspensions of physiological buffer containing HEK293 rDAT cells. Spectrum 1 is the fluorescence emission of physiological buffer alone in the quartz cuvette. Spectrum 2 shows the fluorescence emission of 5 μ*M* Sodium Green™, peak 528 nm, in physiological buffer. Spectrum 3 is the fluorescence emission of 5 μ*M* Sodium Green™, peak 532 nm, after equilibration into HEK293 rDAT cells suspended in physiological buffer containing 150 m*M* Na^+. The vertical lines were added as an aid to illustrate the spectral shift as a result of activation of Sodium Green™ into its Na^+ receptive form and subsequent binding with intracellular Na^+.

Panel B shows the time course of the increase in Sodium Green™ fluorescence after the addition of 10 μM of the pore-forming antibiotic, gramicidin, to a suspension of HEK293 rDAT cells in physiological buffer containing 26 m*M* Na^+ and 2 μ*M* Sodium Green™. The excitation wavelength was 507 nm and the fluorescence emission was monitored at 532 nm. In both panels the "Signal intensity" represents relative fluorescence in arbitrary units of counts/s.


A
Signal Intensity
200000
150000
100000
50000
0
3
2
1
516
536
556
576
596
616
Wavelength (nm)


B
Signal Intensity
30000
27500
25000
0
100
200
300
400
500
600
Time (s)

First, in solution the Na^+ binding to Sodium Green™ occurs in milliseconds (manufacturer's information). Second, the time between the addition of dopamine or gramicidin to dye loaded cells and the observation of a fluorescent signal was observed to be within 5 s (shorter time not measured). Finally, as will be shown later the DAT inhibitors, cocaine and mazindol inhibited the rapid signal from Sodium Green™.

Figure 3 shows that the relationship between $[Na^+]_o$ and the change in relative fluorescent of intracellular Sodium Green™ fits the expected binding isotherm of Na^+ with Sodium Green™. The estimated K_D for the binding of Na^+ with Sodium Green™ agrees well with the literature value of 21 m*M* (from information supplied by the manufacturer, Molecular Probes, Inc. and Amorino and Fox, 1995). In summary these results suggest that: 1) the spectral properties of Sodium Green™ are consistent with expectations that it is able to monitor intracellular Na^+; 2) Na^+ distributes across the HEK293 cell membranes after addition of gramicidin; 3) the distribution of Na^+ achieves apparent equilibrium with timing sufficient to temporally resolve the relationship between dopamine transport and Na^+ accumulation; and 4) that the relative fluorescence of Sodium Green™ follows values of $[Na^+]_i$ according to the expected, literature described binding isotherm for Sodium Green™-Na^+ binding.

Dopamine Transport and Intracellular Accumulation of Na^+ Can be Measured Simultaneously

Trace 1 in Figure 4A shows a typical signal illustrating the increase in relative fluorescence with time observed when a suspension of HEK293 rDAT cells is injected

FIG. 3. **Calibration of Sodium Green™**. The change in relative fluorescence of intracellular Sodium Green™ (2 μM used in the loading step) in gramicidin permeabilized HEK293 rDAT cells as a function [Na^+] and its fit to the expected binding isotherm for Na^+ - Sodium Green™ binding,

$$[Na^+]_i = K_D \frac{(F - F_{\min})}{(F_{\max} - F)}$$

where K_D is the dissociation constant, F is the observed relative fluorescence, F_{min} refers to the minimum fluorescence, and F_{max} the maximal fluorescence. Results are presented as the mean ± SEM, (n = 8). The estimated K_D was found to be 21.2 mM ± 0.4 (SER), r^2 = 0.998.

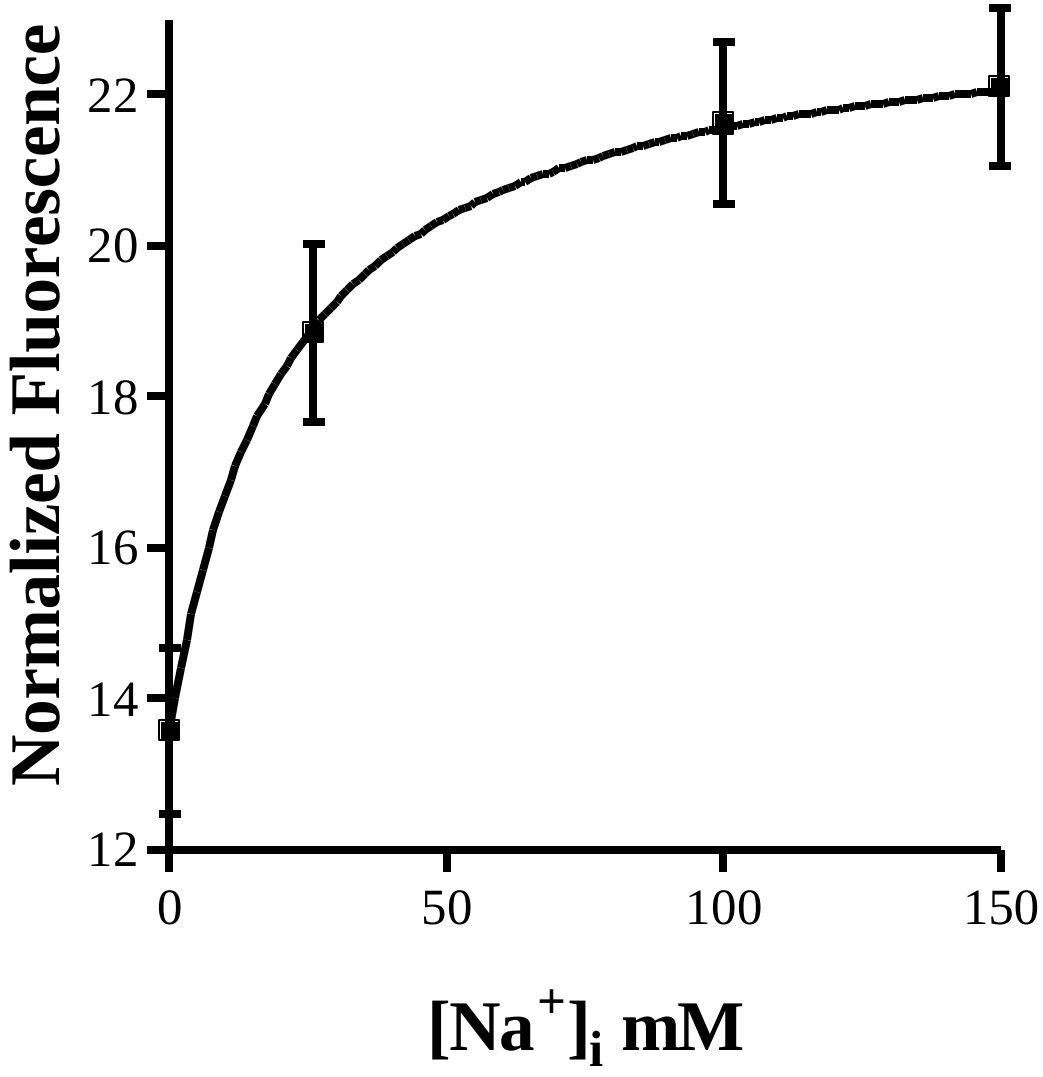
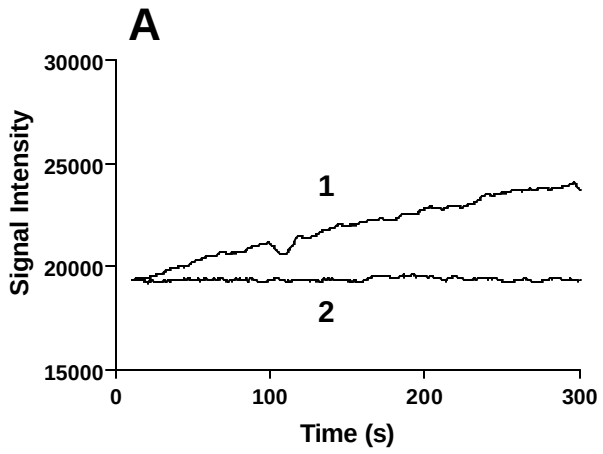
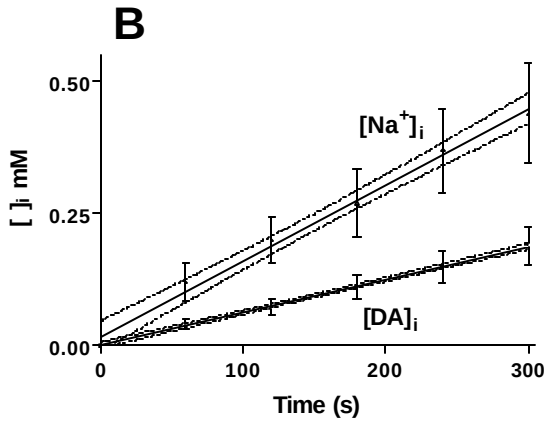
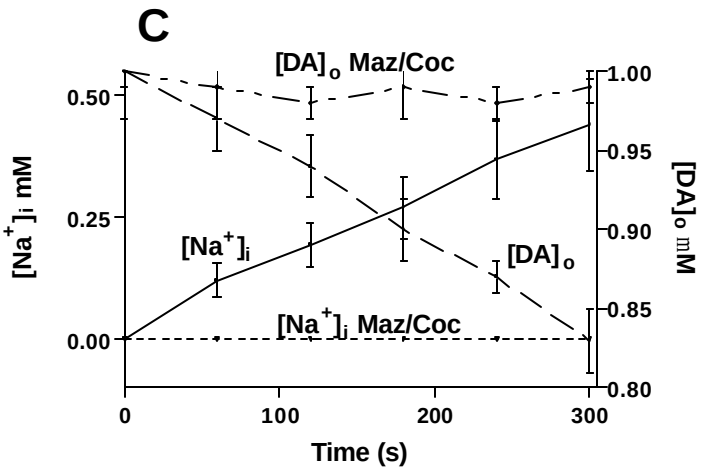

Normalized Fluorescence
22
20
18
16
14
12
0
50
100
150
[Na+]i mM

FIG. 4. The time-course and apparent stoichiometry of the intracellular accumulation of Na^+ during dopamine transport. Panel A shows raw data from two experiments. Line 1 is the increase of fluorescent intensity versus time following a 1 μ*M* dopamine injection into HEK293 rDAT cells loaded with 2 μ*M* Sodium Green™. Line 2 is a separate experiment conducted in the presence of 100 μ*M* cocaine followed by the injection of 1 μ*M* dopamine. For clarity, the scaling of Spectrum 1 was normalized to that of Spectrum 2. Panel B shows the rate of appearance of Na^+ and DA intracellularly at 1 μ*M* dopamine. The slopes of the plots of the increase in $[Na^+]_i$ versus time and $[DA]_i$ versus time were 1.4 ± 0.06 SER X 10^{-5} M/s and 6.2 ± 0.06 SER X 10^{-6} M/s, respectively. Panel C is a summary graph of the time-course of the increase of intracellular Na^+ as a result of the inward movement across the HEK293 rDAT cell membrane concomitantly monitored with transport of 1 μ*M* dopamine from the outside. The excitation wavelength was 507 nm and emission was monitored at 532 nm for 5 min. The inward movement of Na^+ and dopamine is blocked by the addition of 100 μ*M* cocaine or 2 μ*M* mazindol 30 sec prior to the injection of dopamine.


A
30000
25000
20000
15000
Signal Intensity
1
2
0
100
200
300
Time (s)


B
0.50
0.25
0.00
[ ]i mM
[Na+]i
[DA]i
0
100
200
300
Time (s)


C
[DA]o Maz/Coc
0.50
0.25
0.00
[Na+]i mM
[Na+]i
[DA]o
[Na+]i Maz/Coc
1.00
0.95
0.90
0.85
0.80
[DA]o mM
0
100
200
300
Time (s)

with 1 μ*M* dopamine. To confirm that the increase in the Sodium Green™ signal is a function of dopamine transport, 100 μ*M* cocaine was added 30 sec prior to the injection of 1 μ*M* dopamine (Earles and Schenk, 1999). To further confirm that the increase is due to DAT activity, 2 μ*M* mazindol, a more selective inhibitor of dopamine transport (Javitch et al, 1984), was added in separate experiments 30 sec prior to the injection of 1 μ*M* dopamine. Trace 2 in Figure 4A shows that the spectral is blocked in the presence of 100 μ*M* cocaine.

Figure 4B illustrates the time courses of intracellular accumulation of Na^+ and dopamine (at 1.0 μ*M*). For the five minute period analyzed, the rate of $[Na^+]_i$ accumulation was twice (2.3 ± 0.1) that of $[DA]_i$ which is highly suggestive that Na^+ accumulation is time-locked with dopamine transport, and an apparent constant stoichiometric relationship exists between the two co-substrates. The ratio of the slopes was found to be constant even over different temporal regions of the observed signals (data not shown).

Figure 4C is a summary of uptake and inhibition experiments for 1 μ*M* dopamine. In the presence of 2 μ*M* mazindol or 100 μ*M* cocaine, negligible transport of dopamine occurred with no intracellular accumulation of Na^+.

Na^+ Dependence and the Stoichiometry of Na^+-Cotransport with Dopamine

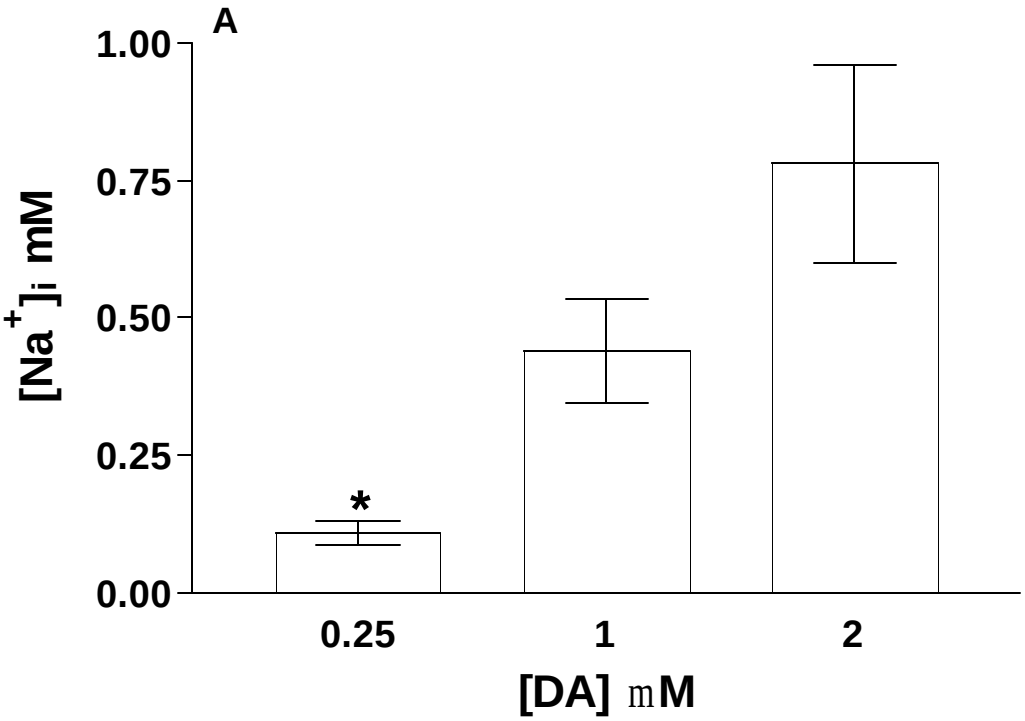
Figure 5 shows that when the measured spectra were evaluated in terms of $[Na^+]_i$ the appearance of the mass of intracellular Na^+ is quantitatively dependent upon the mass of transported dopamine. Three separate values of $[DA]_o$ were evaluated. The resting value of $[Na^+]_i$ was found to be 0.2 ± 0.1 m*M*, n = 24, a value in agreement with Grishin

and Caplan (1998) who also measured Na^+ by flame photometry in their studies of HEK 293 cells. Table 1 shows that for the values of $[DA]_o$ examined, the ratio of Na^+ to dopamine during transport in HEK293 rDAT cells is around 2:1. Results presented in Table 2 show that the ratio of Na^+ to dopamine transported in buffer containing 0 m*M* K^+ was indistinguishable from that observed under the conditions of Table 1 where the buffer contained 3.04 m*M* K^+.

Because Sodium Green™ is a single wavelength dye, there are inherent imprecisions resulting from the inability to correct for small changes in dye concentration occurring during the course of the experiment that can be accounted for with ratiometric fluorescent dyes (Lakowicz, 1999). Further, if the values of precision that are indicated in the data of Sodium Green™ are considered, it may be expected that the error of the stoichiometric estimate may be around 30%. Thus, flame photometry was used as a second method to validate the apparent ≥ 2 to1 stoichiometric ratio between Na^+ and dopamine. Figure 5B shows the results of flame photometric analysis of dopamine stimulated accumulation of Na^+ in HEK293 rDAT cells. The measured stoichiometric ratio of 5 μ*M* dopamine transport was 1.9 ± 0.1 SEM, n = 7, Na^+ per molecule of dopamine.

Panels A and B in Figure 6 illustrate results obtained in similarly conducted experiments in suspensions of HEK293 hDAT cells. The resting value of $[Na^+]_i$ was found to be 0.1 ± 0.1 m*M*, n = 24. Table 1 also contains results similar to those found with HEK293 rDAT cells. The measured ratio of the mass of Na^+ accumulated relative to that of dopamine at 5 μ*M* dopamine, as measured by flame photometry, was 1.9 ± 0.2 SEM, n = 8, Na^+ to dopamine.

FIG. 5. **Quantitative analysis of intracellular Na^+ accumulation by fluorescence and flame photometry in HEK293 rDAT cells**. In Panel A Na^+ accumulation was measured at varying concentrations of dopamine as determined by fluorescence. Dopamine was injected into the cell suspension and followed for 5 min. Statistical differences were tested using repeated measures ANOVA followed by a post-hoc Tukey test. Differences were set at $p = 0.01$. Results at 0.25 μ*M* dopamine relative to those at 1 μ*M* and 2 μ*M*, respectively, were statistically different. The results at 1 μ*M* dopamine versus those at 2 μ*M* dopamine were not statistically different ($p > 0.05$). Results are presented as the mean ± SEM, n = 5. In Panel B Na^+ accumulation was measured by flame. The suspension of cells was injected with 5 μ*M* dopamine and incubated for 15 min. The bar "Control" represents results from samples of cells (with no dopamine) to determine basal levels of Na^+ within cell samples. The bar "Cocaine" shows the effect of the addition of 100 μ*M* of cocaine, 30 sec prior to the injection of 5 μ*M* dopamine. The bars "1 Na^+" and "2 Na^+" are predictions of the mass of Na^+ expected in the samples based upon the amount of Na^+ present in the control plus the amount expected due to the average amount of dopamine taken up, 73.5 ± 3.5%, n = 7. The 5 μ*M* dopamine bar shows that accumulation of Na^+ can be accounted for by the transport of 2 Na^+ for each molecule of dopamine transported. Statistical differences were tested using repeated measures ANOVA followed by a post-hoc Tukey test. Differences were set at $p = 0.01$. The result at 5 μ*M* dopamine versus "Control", "Cocaine" and "1 Na^+" respectively, were statistically different. The result at 5 μ*M* dopamine versus "2 Na^+" was not statistically different ($p > 0.05$). Numerical values are presented as the mean ± SEM, n = 7.


A
1.00
0.75
0.50
0.25
0.00
[Na+]i mM
*
0.25
1
2
[DA] mM

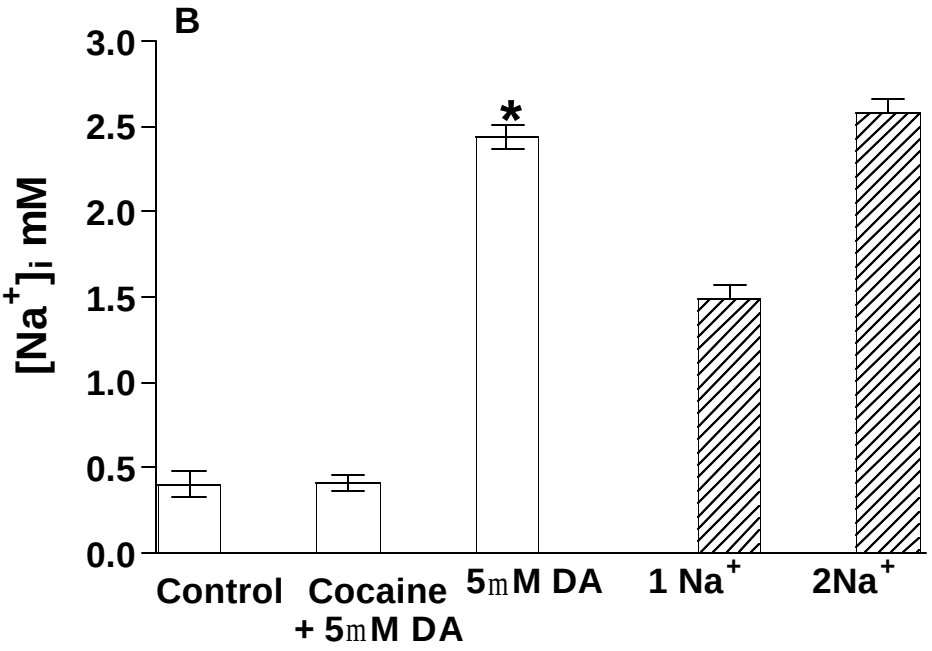

B
3.0
2.5
2.0
1.5
1.0
0.5
0.0
[Na+]i mM
*
Control
Cocaine + 5mM DA
5mM DA
1 Na+
2Na+

Table 1: *Ratios[a] of the number of moles of intracellularly accumulated Na^+ to the number of moles of dopamine taken up by rDAT HEK cells and hDAT HEK cells in physiological buffer containing 150 mM Na^+*

rDAT HEK Cells

Time Point	**$[DA]_o$ = 0.25 mM**	**$[DA]_o$ = 1.0 mM**	**$[DA]_o$ = 2.0 mM**
1 min	2.3 ± 0.1	2.7 ± 0.2	2.2 ± 0.3
2 min	2.8 ± 0.1	2.5 ± 0.2	2.5 ± 0.2
3 min	2.6 ± 0.1	2.4 ± 0.1	2.6 ± 0.3
4 min	3.0 ± 0.1	2.4 ± 0.2	3.0 ± 0.3
5 min	2.7 ± 0.2	2.3 ± 0.1	2.8 ± 0.2

Overall average = 2.6 ± 0.2 SD, n = 15

hDAT HEK Cells

Time Point	**$[DA]_o$ = 0.25 mM**	**$[DA]_o$ = 1.0 mM**	**$[DA]_o$ = 2.0 mM**
1 min	2.4 ± 0.2	2.4 ± 0.1	2.2 ± 0.1
2 min	2.2 ± 0.1	2.7 ± 0.2	2.7 ± 0.1
3 min	2.5 ± 0.2	2.5 ± 0.2	2.7 ± 0.2
4min	2.7 ± 0.1	2.7 ± 0.1	2.4 ± 0.1
5 min	2.6 ± 0.1	2.7 ± 0.1	2.4 ± 0.1

Overall average = 2.5 ± 0.2 SD, n = 15

[a]Average values ± SEM

Table 2: *Ratios[a] of the number of moles of intracellularly accumulated Na^+ to the number of moles of dopamine taken up by rDAT HEK cells in physiological buffer containing 0 mM K^+ following the addition of 1.0 mM dopamine*

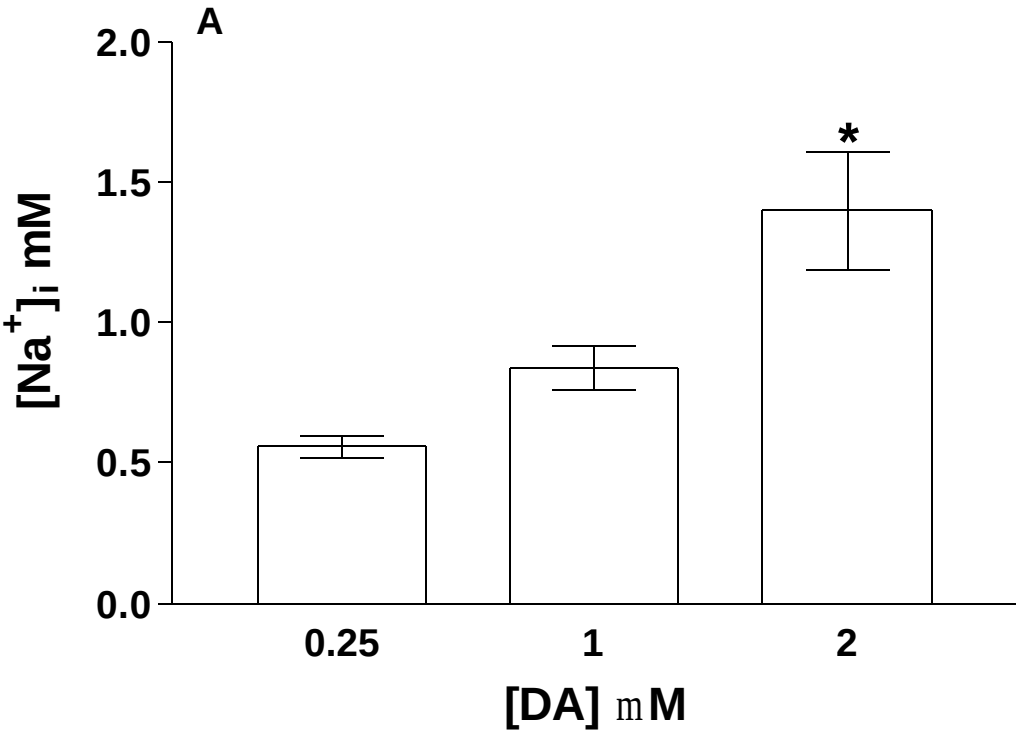
Time (min)	Ratio
1	2.4 ± 0.1
2	2.4 ± 0.4
3	2.3 ± 0.2
4	2.4 ± 0.1
5	2.4 ± 0.2

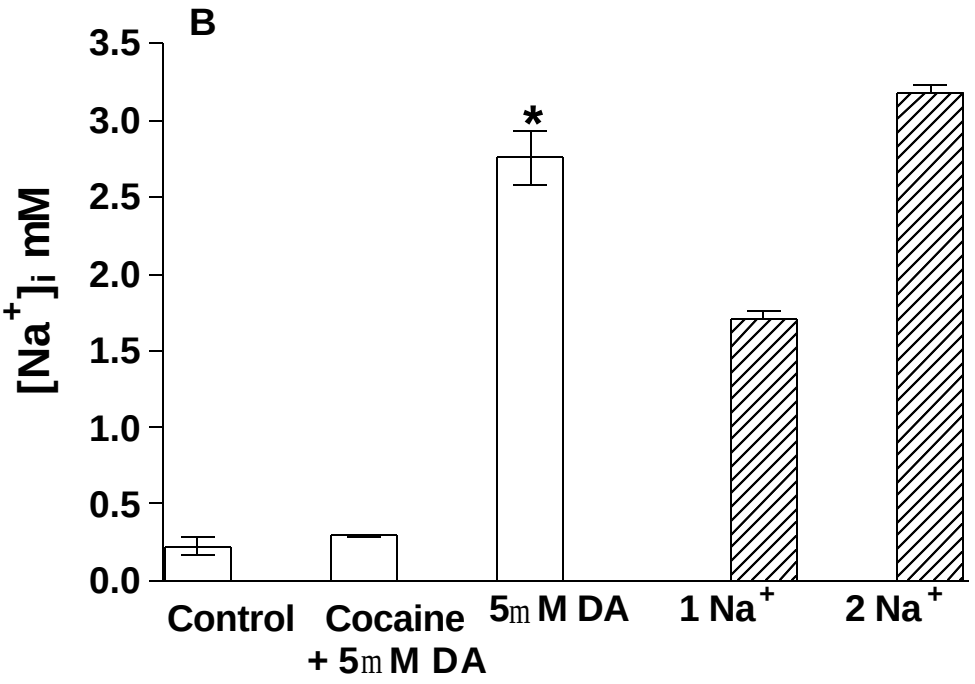
Overall ratio = 2.4 ± 0.5

[a]Average values ± SEM

To ensure the validity of applying the *in situ* Sodium Green™ calibration for rDAT cells to calculations with cells expressing hDAT, experiments were conducted at each [Na^+] using hDAT cells and compared to previous results obtained using rDAT cells. There was no statistical difference between the Na^+ dependent signals obtained for hDAT cells and rDAT cells which was the expected result given that the cell lines were identical except for the transporter expressed (data not shown).

FIG. 6. **Quantitative analysis of intracellular Na^+ accumulation by fluorescence and flame photometry in HEK293 hDAT cells**. In Panel A Na^+ accumulation was measured at varying concentrations of dopamine as determined by fluorescence. Dopamine was injected into the cell suspension and followed for 5 min. Statistical differences were tested using repeated measures ANOVA followed by a post-hoc Tukey test. Differences were set at $p = 0.01$. The results at 2 μ*M* dopamine relative to those at 0.25 μ*M* dopamine and 1 μ*M* dopamine, respectively, were statistically different. The results at 0.25 μ*M* dopamine versus 1 μ*M* dopamine were not statistically different ($p > 0.05$). Values are presented as the mean ± SEM, $n = 5$. In Panel B Na^+ accumulation in cells was measured by flame photometry. The suspension of cells was injected with 5 μ*M* dopamine and incubated for 15 min. The bar "Control" represents results from samples of cells (with no dopamine) to determine basal levels of Na^+ within cell samples. The bar "Cocaine" shows the effect of the addition of 100 μ*M* of cocaine, 30 sec prior to the injection of 5 μ*M* dopamine. The bars "1 Na^+" and "2 Na^+" are predictions of the mass of Na^+ expected in the samples based upon the amount of Na^+ present in the control plus the amount expected due to the average amount of dopamine taken up, 76.6 ± 3.0%, $n = 8$. The 5 μ*M* dopamine bar shows that accumulation of Na^+ can be accounted for by the transport of 2 Na^+ for each molecule of dopamine transported. Statistical differences were tested using repeated measures ANOVA followed by a post-hoc Tukey test. Differences were set at $p = 0.01$. The result with 5 μ*M* dopamine versus "Cocaine", "Control" and "1 Na^+" respectively, were statistically different. The result at 5 μ*M* dopamine versus "2 Na^+" was not statistically different ($p > 0.05$). Numerical values are presented as the mean ± SEM, $n = 8$.


A
2.0
1.5
1.0
0.5
0.0
[Na+]i mM
*
0.25
1
2
[DA] mM


B
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
[Na+]i mM
*
Control
Cocaine + 5mM DA
5mM DA
1 Na+
2 Na+

DISCUSSION

The general kinetic mechanism of DAT in native preparations of striatum and nucleus accumbens from the rat has been proposed to involve Na^+ and Cl^- as cosubstrates (Harris and Baldessarini, 1972; Holz and Coyle, 1974; Kuhar and Zarbin, 1978). The DAT appears to follow a sequential kinetic mechanism in which Na^+ and dopamine randomly bind first to the external face of the transporter protein followed by the binding of Cl^- prior to the conformational change in DAT protein mediating translocation (McElvain and Schenk, 1992; Wheeler et al., 1993; Povlock and Schenk, 1997). In HEK cells expressing hDAT the process is also sequential except that Na^+ binds before dopamine with Cl^- binding last prior to translocation (Chen et al., 1999; Earles and Schenk, 1999). The stoichiometry of only a few Na^+ and Cl^- dependent transporters has been confirmed by measurements of mass accumulation of substrates (for examples and discussions see Crane, 1960; 1977; Heinz, 1972; Henius and Laris, 1979; Turner and Moran, 1982; Stein, 1986; 1989; Weiss, 1996; Krupka, 1993; 1998; Risso et al., 1996). Here the accumulation of intracellular Na^+ cotransported with dopamine by rDAT and hDAT expressed in HEK cells was measured on a concentration as well as a mass basis and found to have a stoichiometric ratio of 2 : 1, Na^+ to dopamine. This is an important finding for DAT because it confirms the Na^+/dopamine relationship in terms of stoichiometry as opposed to reaction orders of two, highly suggestive of a stoichiometric ratio (McElvain and Schenk, 1992; Wheeler et al., 1993, 1994; Povlock and Schenk, 1997; Gu et al., 1994; Pifl et al., 1997; Earles and Schenk, 1999), but not definitive (Stein, 1990; Rudnick and Clark, 1993). The kinetic results cited, though strongly suggestive of a stoichiometry greater than unity for Na^+, also could be accounted for by

one or two Na^+ acting as essential activators of DAT without being transported or even the case where two Na^+ are required for activation of the transporter with only one Na^+ involved in the transport cycle (translocation) (Segel, 1993; Clark and Rudnick, 1993; Povlock and Schenk, 1997; Earles and Schenk, 1999). The results presented here in combination with previous studies in HEK expression systems (Earles and Schenk, 1999) suggest that Na^+ is an essential substrate in the DAT-mediated translocation of dopamine, that is it is required for DAT activity and participates as a co-substrate during translocation. It should be noted here that *stoichiometry* refers to the ratio of Na^+ to dopamine during transport. The actual number of $Na^{+'}$s and dopamine molecules transported in a single transport cycle (or during catalytic turnover) for a single DAT molecule is unknown. Furthermore, the analyses presented did not take into account the putative effects of the activity of Na^+/K^+ ATPase on values of $[Na^+]_i$. On first consideration this may be expected to bias our results toward lower stoichiometric values for Na^+. An experimental observation and a couple literature lines of argument reduce the likelihood that the apparent stoichiometric ratio of Na^+ to dopamine is affected by Na^+/K^+ ATPase activity. First, in experiments conducted here under 0 m*M* K^+' the ratio of Na^+ to dopamine (2 to 1) was no different from that observed under conditions of 3.04m*M* K^+ externally' suggesting that the Na^+/K^+ ATPase does not play a role in reducing the $[Na^+]_i$ during the experiment. Experiments with ouabain at 100 μ*M* were not helpful in that the onset of the effect of changes in intracellular and extracellular Na^+ and K^+ were not observed until after incubations for periods longer (> 7 minutes) than the duration of the dopamine transport experiment (data not shown) and were not complete until > 33 min had elapsed. Second, even with strong cooperative activity of the ATPase

toward Na^+, a coefficient of 3 and, a K_{Na} of 10 m*M* to 15 m*M* (Akar, 1986; Sachs 1977), the maximal value of $[Na^+]_i$ observed here would only saturate the ATPase at ≤ 5%. Finally, it has been shown that dopamine inhibits the Na^+/K^+ ATPase in kidney cells (Svennilson and Aperia, 1999). Thus, the Na^+/K^+ ATPase would be expected to have little influence on our results.

Further, special problems may exist when reaction orders are relied upon solely as indictors of stoichiometry. Chen et al (1999) questioned the veracity of reaction order estimations by showing that different ion substitutes for Na^+ (each decreasing the velocity of dopamine transport) in reaction order experiments can result in different apparent reaction order dependencies even under conditions in which other investigators, with the same ion substitute, find only a single reaction order relationship. Some equilibrium binding models (Li and Reith, 1999; 2000) of DAT are consistent also with a 2 to 1 relationship between Na^+ and some inhibitor and substrate binding sites. These binding models could be considered to represent the pre-steady state of DAT, however, exact delineation of substrate transport stoichiometry in these non-catalytic preparations is not possible. The reason that this mechanistic issue is important physiologically is that the neuronal DAT protein operates asymmetrically and electrogenically concentrating dopamine into the intracellular compartment at concentration values in the 10^6 to 10^7 range over those in the extracellular phase (Earles and Schenk, 1999). A significant energy source for this accumulation, and indeed for Na^+-dependent transporters in general (Schafer, 1972; Jacquez, 1972; Crane, 1977; Stein, 1989; 1990; Weiss, 1996), is thought to be the external to internal Na^+ concentration gradient and the amount of energy that results from this gradient coupling. The energy depends on the stoichiometric

coupling ratio between the apparent driven substrate, dopamine, and the driving substrate, Na^+ (Stein, 1989; 1990). In addition, more than one group has shown that some inhibitors of DAT activity may function by altering Na^+ binding at or translocation of Na^+ by DAT (and other biogenic amine transporters) (Bönisch, 1986; McElvain and Schenk, 1992; Wheeler et al., 1994; Li and Reith, 1999; 2000) making this phenomenon a potentially important issue in the pharmacology of DAT.

An energy balance argument can be made to assess whether the proposed stoichiometry of two Na^+ to one dopamine can account significantly for the energy requirement for dopamine accumulation by DAT. The energy required for the range of accumulation observed by DAT can be estimated from the chemical potential of a 10^6 to 10^7 concentration gradient which is in the range 8.5 to 9.9 kcal/mole. If one assumes that the $[Na^+]_i$ is ~ 0.2 m*M* and the $[Na^+]_o$ is ~ 150 m*M*, as expected from the results of experiments conducted here, and the coupling ratio (stoichiometry) is unity then the energy obtained from this condition would be about 4.5 kcal/mole, a value far from that required. However, if the stoichiometry is two, then the amount of energy available would be increased by the ratio of the squares of the two values for Na^+ and the energy available would be increased to 9.0 kcal/mole. When the energy contributed by the transmembrane potential, -60 mv (Hoffman et al., 1999), and unequal distribution of Cl^- is added to this value the energy available for coupling to transport is about 13 kcal/mole, a value very close to that required.

Results of electrophysiological studies suggest that the DAT under certain conditions (Sitte et al., 1998; Sonders et al., 1997; Sonders and Amara, 1998) as well as other transporters (Risso et al, 1996 and for reviews see Lester, 1994; 1996) can also act

as channels for cations, and the stoichiometric coupling of Na^+ and dopamine may not be operational under these conditions. Thus, the results of the work here confirm that a stoichiometric relationship between dopamine and Na^+ exists during translocation of dopamine. Taken with the results of the cited electrophysiological work above our results also support the concept of a functional role for DAT which includes both the stoichiometric (traditionally viewed) functioning of transporters as well as a functional mode reminiscent of ion channels at least with respect to Na^+. The nature of the interaction between dopamine and DAT occurring during the channel mode is unknown at present. Sonders and Amara (1998) have suggested that the channel behavior might serve to alter neuronal membrane excitability. In addition to this hypothesis the channel behavior cited might serve as regulatory mechanism for the control of dopamine transport. If it is assumed that the coupling to Na^+ serves as an energy source for transport of dopamine and the coupling is, under some conditions, tightly regulated at a stoichiometry of two then any process which alters this coupling could change the DAT-mediated movement of dopamine. In the channel mode DAT could essentially short circuit the Na^+ driving force by allowing a non-stoichiometric flow of Na^+ across the membrane. This may represent a dynamic control of slippage in transport systems as described by Stein (1986) where the coupling of driven (dopamine) and driving (Na^+) varies according to the state of a transporter. Thus in addition to altering the transmembrane potential as proposed by Sonders and Amara (1998) this mechanism could serve to reduce the transport of dopamine by modulating co-substrate involvement. Further work will be required to validate these hypotheses.

In summary, it has been shown that the Na^+ to dopamine stoichiometric ratio during transport by the DAT is two to one, and with more certain knowledge of this quantitative relationship, hypotheses can be formulated to guide future experimentation in the apparent transporter/channel dual nature of DAT.

Acknowledgment: This work was supported by the State of Washington and a grant (RO1 DA07384) to JOS from the National Institute on Drug Abuse (NIDA). PDS was supported by the United States Army, and JOS is also a recipient of NIDA Independent Scientist Award, KO2 00184. We would like to thank Dr. Jeremy Lessmann for his spectroscopic expertise and assistance during the course of this work.

REFERENCES

Aker T. (1986) The function of Na^+,K^+-ATPase and its importance for drug action, in *Cardiac Glycosides 1785-1985 Biochemistry, Pharmacology, and Clinical Relevance* (Erdmann E., Greef, K., and Skou J. C., eds.), 19-25. Springer-Verlag, Berlin.

Amara S. G. and Kuhar M. J. (1993) Neurotransmitter transporters: recent progress. *Ann. Rev. Neurosci.* **16**, 73-93.

Amorino G. P. and Fox M. H. (1995) Intracellular Na^+ measurements using sodium green tetraacetate with flow cytometry. *Cytometry* **21**, 248-256.

Bogdanski D. F. (1976) Mechanisms of transport for the uptake and release of biogenic amines in nerve endings. *Adv Exp Med Biol* **69**, 291-305.

Bönisch H. (1986) The role of co-transported sodium in the effect of indirectly acting sympathomimetic amines. *Naunyn-Schmiedeberg's Arch Pharmacol* **332**, 135-441.

Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye binding. *Anal. Biochem*. **72**, 248-254.

Chen N., Trowbridge C.G., and Justice J.B. (1999) Cationic modulation of human dopamine transporter: dopamine uptake and inhibition of dopamine uptake. *J. Pharmacol. Exp. Thera*. **290**, 940-949.

Crane R. K. (1960) Intestinal absorption of sugars. *Physiol. Rev*. **40**, 789-825.

Crane R. K. (1977) The gradient hypothesis and other models of carrier mediated active transport. *Rev. Physiol. Biochem. Pharmacol*. **78**, 99-159.

Earles C. and Schenk J. O. (1999) Multisubstrate mechanism for the inward transport of dopamine by the human dopamine transporter expressed in HEK cells and it inhibition by cocaine. *Synapse* **33**, 230-238.

Earles C., Wayment H., Green M., and Schenk J. O. (1998) Resolution of biogenic amine transporter kinetics by rotating disk electrode voltammetry: methodology and mechanistic interpretations. *Meth Enzymol* **296**, 660-675.

Earles C. and Schenk J. O. (1998) Rotating disk electrode voltammetric measurements of dopamine transporter activity: an analytical evaluation. *Anal. Biochem*. **264**, 191-198.

Friedman J. E. and Haddad G. G. (1994) Anoxia induces an increase in intracellular sodium in rat central neurons in culture. *Brain Res* **663**, 329-334.

Garrahan P. J. and Glyn I. M. (19667) *J. Physiol. (London)* **192**, 189-216.

Graefe, K-H. (1988) Ion dependence of neuronal amine transport, in *Progress In Catecholamine Research, Part A: Basic Aspects and Peripheral Mechanisms* (Dalström A., Belmaker R. H., and Sandler M., eds) pp. 131-134. Alan R. Liss, NY.

Grishin A. V. and Caplan M. J. (1998) ATP1AL1, a Member of the non-gastric H,K-ATPase Family, Functions as a Sodium Pump. *J. Biol. Chem*. **273**, 27772-27778.

Grynkiewicz G, Poenie M, and Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem*. **260**, 3440-3448.

Gu H., Wall S.G., and Rudnick G. (1994) Stable expression of biogenic amine transporters reveals differences in inhibitor sensitivity, kinetics, and ion dependence. *J. Biol. Chem*. **269**, 7124-7130.

Harootunian A. T., Kao J. P. Y., Eckert B. K., and Tsien R. Y. (1989) Fluorescence ratio imaging of cytosolic free Na+ in individual fibroblasts and lymphocytes. *J. Biol. Chem*. **264**, 19458-19467.

Harris J. F. and Baldessarini R. J. (1972) The uptake of [^{3}H]dopamine by homogenates of rat corpus striatum: effects of cations. *Life Sci* **13**, 303-313.

Heinz E., ed (1972) Na-linked transport of organic solutes: the coupling between electrolyte and nonelectrolyte transport in cells. Berlin: Springer-Verlag.

Henius G. V. and Laris P. C. (1979) The Na^+ gradient hypothesis in cytoplasts derived from Ehrlich ascites tumor cells. *Biochim. Biophys. Res. Commun*. **91**, 1430-1436.

Hodgkin A. L. and Keynes R. D. (1954) Movement of cations in the recovery of nerves. *Symp. Soc. Exp. Biol.* **8**, 423-437.

Hoffman A. F., Zahniser N. R., Lupica C. R., and Gerhardt G. A. (1999) Voltage-dependency of the dopamine transporter in the rat substantia nigra. *Neurosci. Lett*. **260**, 105-108.

Holz K. W. and Coyle J. T. (1974) The effects of various salts, temperature, and alkaloids, veratridine and batrachotoxin on the uptake of [^{3}H]-dopamine into synaptosomes from rat striatum. *Mol. Pharm*. **10**, 746-758.

Jacquez J. A. (1972) Ion gradient hypotheses and the energy requirement for active transport of amino acids. In: Na-linked transport of organic solutes: the coupling between electrolyte and no electrolyte transport in cells (Heinz E, ed), pp 4-14 Berlin: Springer-Verlag.

Javitch J. A., Blaustein R. O., and Snyder S. H. (1984) [^{3}H]-Mazindol binding associated with neuronal; dopamine and norepinephrine uptake sites. *Molec. Pharmacol*. **26**, 35-44.

Krueger B. K. (1990) Kinetics and block of dopamine uptake in synaptosomes from rat caudate nucleus. *J. Neurochem*. **55**, 260-267.

Krupka R. M. (1993) Coupling mechanisms in active transport. *Biochim Biophys Acta* **1183**, 105-113.

Krupka R. M. (1998) Channeling free energy into work in biological processes. *Exp. Physiol*. **83**, 243-251.

Kuhar M. J. and Zarbin M. A. (1978) Synaptosomal transport: a chloride dependence for choline, GABA, glycine, and several other compounds. *J. Neurochem*. **31**, 251-256.

Lakowicz J. D. (1999) Fluorescence Sensing, Chapter 9 in *Principles of Fluorescence Spectroscopy*, 2nd ed., Kluwer Academic/Plenum Press, NY pp. 531-572.

Lester H. A., Cao Y., and Mager S. (1996) Listening to neurotransmitter transporters. *Neuron* **17**, 807-810.

Lester H. A., Mager S., Quick M. W., and Corey J. L. (1994) Permeation properties of neurotransmitter transporters. *Ann. Rev. Pharmacol. Toxicol*. **34**, 219-249.

Li L.-H. and Reith M. E.A. (1999) Modeling of the interaction of Na^+ and K^+ with binding of dopamine and [^{3}H]WIN 35,428 to the human dopamine transporter. *J. Neurochem*. **72**, 1095-1109.

Li L.-H. and Reith M. E. A. (2000) Interaction of Na^+, K^+, and Cl^- with the binding of amphetamine, octopamine, and tyramine to the human dopamine transporter. *J. Neurochem*. **74**, 1538-1552.

McElvain J. S. and Schenk J. O. (1992) A multisubstrate mechanism for uptake of striatal dopamine uptake and its inhibition by cocaine. *Biochem. Pharmacol*. **43**, 2189-2199.

Meiergerd S. M. and Schenk J. O. (1995) Measurement of the time-resolved kinetics of biogenic amine release and transporter activity by rotating disk electrode voltammetry in vitro. In: *Voltammetric methods in brain systems, Neuromethods , Vol. 27* (Boulton AA, Baker G, and Adams RN, eds) pp 305-337. Totowa NJ: Human Press.

Pifl C., Agneter E., Drobny H., Reither H., and Singer E. A. (1997) Induction by low Na^+ or Cl^- of cocaine sensitive carrier-mediated efflux of amines from cells transfected with cloned human catecholamine transporters *Br. J. Pharmacol*. **121**, 205-212.

Povlock S. L. and Schenk J. O. (1997) A multisubstrate kinetic mechanism of dopamine transport in the nucleus accumbens and its inhibition by cocaine. *J. Neurochem*. **69**, 1093-1105.

Risso S., DeFlice L. J., and Blakely R. D. (1996) Sodium-dependent GABA-induced currents in GAT1 transfected HeLa cells. *J. Physiol*. (Lond) **490**, 691-702.

Rudnick G. and Clark J. (1993) From synapse to vesicle: the reuptake and storage of biogenic amine neurotransmitters. *Biochim Biophys Acta* **1144**, 249-263.

Sachs J. R (1977) Kinetic evaluation of the Na/K pump reaction mechanism. *J. Physiol. (London)* **273**, 489-514.

Schafer J. A. (1972) An examination of the energetic adequacy of the ion gradient hypothesis for nonelectrolyte transport. In: Na-linked transport of organic solutes: the coupling between electrolyte and non electrolyte transport in cells. (Heinz E ed.) pp 68-83 Berlin: Springer-Verlag.

Schumacher P. D., Lessmann J. J., Schenk J. O. (2000) The stoichiometry of the accumulation of Na^+ via the dopamine transporter is two Na^+'s to one dopamine. *Neurosci. Abstr.* **26**, 438.9.

Segel I. H., ed (1993) *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems.* pp 340-385 New York: John Wiley & Sons, Inc.

Sitte H. H., Huck S., Reither H., Boehm S., Singer E. A., and Pifl C. (1998) Carrier-mediated release, transport rates, and charge transfer induced by amphetamine, tyramine, and dopamine in mammalian cells transfected with the human dopamine transporter. *J. Neurochem*. **71**, 1289-1297.

Sonders M. S. and Amara S. G. (1998) Channels in transporters. *Curr. Op. Neurobiol.* **6**, 294-302.

Sonders M. S., Zhu S. –J., Zahniser N. R., Kavanaugh M. P., and Amara S. G. (1997) Multiple ionic conductances of the human dopamine transporter: actions of dopamine and psychostimulants *J. Neurosci*. **17**, 960-974.

Stein W. D. (1986) *Transport and Diffusion across Cell Membranes*. San Diego, CA: Academic Press, Harcourt Brace Jovanovitch, Publishers.

Stein W. D. (1989) Kinetics of transport: analyzing, testing, and characterizing models using kinetic approaches. *Adv. Enzymol*. **171**, 23-62.

Stein W. D. (1990) *Channels, Carriers, and Pumps: An Introduction to Membrane Transport*. pp 127-171. San Diego, CA: Academic Press, Harcourt Brace Jovanovitch, Publishers, pp. 127-171.

Steinbach H.B. (1954) The regulation of sodium and potassium in muscle fibres. *Symp. Soc. Exp. Biol.* **8**, 438-452.

Svennilson J. and Aperia A. (1999) Dopamine in the developing kidney. *Int. J. Biol*. **43**, 441-443.

Szmacinski H. and Lakowicz J. R. (1997) Sodium green as a potential probe for intracellular sodium imaging based on fluorescence lifetime. *Anal. Biochem*. **250**, 131-138.

Turner R. J. and Moran A. (1982) Stoichiometric studies of the renal outer cortical brush border membrane D-glucose transporter. *J. Memb. Biol*. **67**, 73-80.

Weiss T. F. (1996). Carrier-mediated transport. In: *Cellular Biophysics, Volume 1: Transport*. pp 333-447 Cambridge, MA: A Bradford Book, The MIT Press.

Wheeler D. D., Edward A. M., Chapman B. M., and Ondo J. G. (1993) A model of the sodium-dependence of dopamine uptake in rat striatal synaptosomes. *Neurochem. Res*. **18**, 927-936.

Wheeler D. D., Edward A. M., Chapman B. M., and Ondo J. G. (1994) Effects of cocaine on sodium dependent dopamine uptake in rat striatal synaptosomes. *Neurochem. Res*. **19**, 49-56.